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# ANTONIE VAN LEEUWENHOEK

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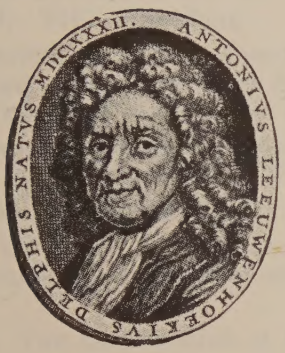


**Official organ of the**

**„NEDERLANDSCHE VEREENIGING VOOR MICROBIOLOGIE"**

**(Netherlands Society of Microbiology)**



SWETS EN ZEITLINGER
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ, AMSTERDAM

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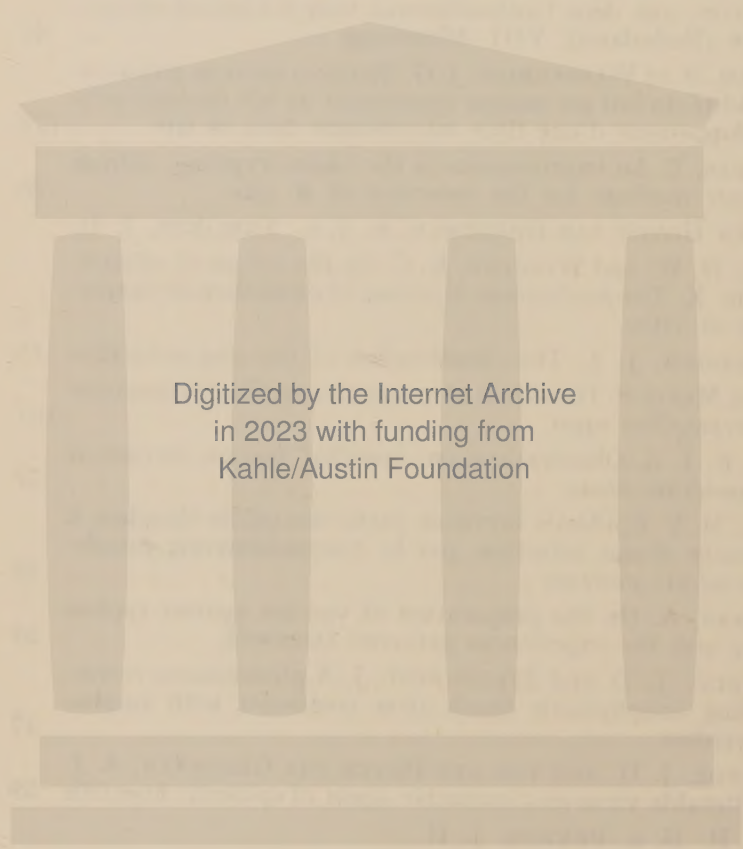

(From the Laboratory of Hygiene of the University, Utrecht).

# ON THE ACTION OF SULFANILAMIDE

## X. THE MECHANISM OF ACTION OF SULFANILAMIDE DERIVATIVES IN VITRO [1])

by

**H. W. JULIUS** and **K. C. WINKLER**

(Received May 27, 1943).

### A. INTRODUCTION.

The mechanism of action of sulfanilamide in vitro differs widely from the action of germicides in general, which the following characteristic features render evident:

*a*) sulfanilamide is not directly bactericidal; most bacteria survive for 24 hours and longer even in excessive concentrations. A RIDEAL-WALKER coefficient cannot be determined.

*b*) the action of the drug occurs only in growing cultures, inoculated with a not over large number of bacteria and does not become manifest until some hours of normal growth have elapsed ("lag time of action" c.f. fig. 2 and fig. 3).

*c*) addition of blood or serum, so deleterious on the effect of other desinfectants seems not to decrease this sulfanilamide action, but even to enhance it under certain circumstances.

*d*) the action of sulfanilamide is counteracted (specifically?) by very small concentrations of p. aminobenzoic acid.

With regard to the mechanism of action of the compounds of sulfanilamide it soon became evident that all drugs of the sulfamide-group actually show the same features and it is quite evident that in vitro at least, the mechanism of action within this group is essentially the same.

Still it is beyond doubt that some of the compounds of sulfanilamide (sulfapyridine, sulfathiazol a.o.) are more potent drugs than sulfanilamide itself. It is one of the objects of this paper to contribute to our knowledge of this problem.

In no. III of this series one of us (2) showed, for sulfapyridine, that apart from the sulfanilamide-action, so far as in vivo conditions are concerned, another activity must be present in the molecule. Some evidence was produced, that this activity involved the defence of the host, though it was not possible to elucidate, in which way this might be realised.

A better quantitative analysis of the in vitro conditions showed, however, that also in vitro there does exist a distinct quantitative difference in the activity of sulfanilamide and its various compounds, thus providing an additional explanation for differences in vivo. Differences in toxicity,

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[1]) Ninth communication: K. C. WINKLER, Antonie van Leeuwenhoek **9**, 115, 1943.

solubility and many of the like of course also have to be taken in account for explaining the greater effectiveness of some of the heterocyclic derivatives in vivo.

In this paper we will limit ourselves strictly to the in vitro action of sulfanilamide and its derivatives. The mechanism of action being the same, a higher activity in vitro of the derivatives as compared with sulfanilamide can only mean: the same activity in lower concentrations. Indeed in growth experiments the difference between sulfapyridine and sulfanilamide appears to be no other than a difference in concentration (Exp. I).

The question thus arises: why smaller amounts of sulfapyridine are equally active as sulfanilamide, the mechanism of action being the same.

B. Activity and adsorption.

p.Aminobenzoic acid inhibits the action of sulfanilamide to such an

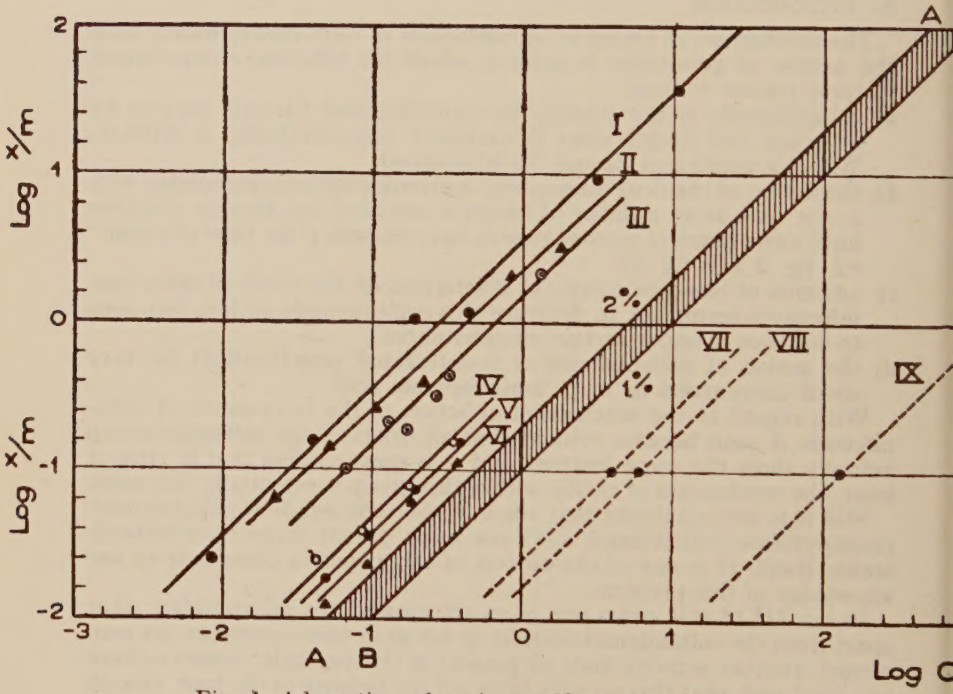



Fig. 1. Adsorption of various sulfonamides by *B. coli*.

log x/m = adsorbed quantity in m.mol. per litre of bacteria.
log $C_1$ = equilibrium concentration in m.mol. per litre.
I. p. aminobenzoic acid
II. methylsulfathiazol
III. sulfathiazol
IV. 2-sulfanilamido-4-methylpyrimidine
V. sulfapyridine
VI. 5-sulfanilamido-4-methylpyrimidine
A—A. limit of measurableness for an experimental error of 2 %
B—B. limit of measurableness for an experimental error of 1 %
VII. supposed curve for both the sulfanilamido-pyrazoles
VIII. supposed curve for sulfanilamide
IX. supposed curve for sulfanilic acid

extent that the counteracting concentration in the case of *B. coli* is 1200 times smaller than the sulfanilamide concentration which was active (3). As, in the end one molecule of p.aminobenzoic acid can compete with merely one molecule of sulfanilamide, the difference in active concentrations has to be explained; f.i. by an adsorption process. That means, that the adsorbed quantity of both substances should be equal at very different concentrations. In fact we found (3) that p.aminobenzoic acid is actively adsorbed by *B. coli*, whilst sulfanilamide (within the experimental error) is not.

Considering this fact it might be supposed that the greater activity of sulfapyridine (and other derivatives) also could be due to a better adsorption in the bacteria.

To produce corroborating evidence we studied in how far the activity of thirteen sulfanilamide compounds for *B. coli* was related to their adsorption to this organism.

The activity was studied by adding various concentrations of each drug to a synthetic medium, inoculated with *B. coli* and determining the s m a l l e s t e f f e c t i v e c o n c e n t r a t i o n. The viable count was used as a measure in preference to the turbidity which is often used in this kind of experiments, but which is not sensitive enough for smaller numbers of bacteria and does not differentiate living bacteria from dead. Moreover the form of the growth curves (f.i. fig. 2) shows that the essentials of the process will be missed if turbidity is used as a measure.

The a d s o r p t i o n of sulfanilamide or its derivatives was determined by adding various concentrations of the used drug to concentrated suspensions of *B. coli* and after centrifuging, estimating the concentration of the drug in the supernate, by diazotization and coupling to oxychinoline.

Experiment I for instance bears on the determination of the activity of sulfapyridine, whilst in Experiment II some instances of the determination of adsorption are given. The results are collected in table I. In table I, columm a, the minimal active concentrations of the studied drugs are given, whereas the relative activity with regard to sulfanilamide can be found in column b. It is evident for instance from these figures that sulfapyridine is 30 times, sulfathiazol 75 times more active than sulfanilamide and so on.

The results of the adsorption experiments are presented in fig. 1. The log of the adsorbed quantity per unit of bacteria (log x/m) is plotted against the log of the equilibrium-concentration in m.moles per l. All curves appear to be parallel to each other with an inclination of 45°. p.Aminobenzoic acid appears to be adsorbed better than any of the sulfanilamides under discussion. Ordering the latter in the order of sequence of decreasing adsorption the following series is obtained:

Methylsulfathiazol>sulfathiazol>2-sulfanilamido-4-methylpyrimidine>sulfapyridine>5-sulfanilamido-4-methylpyrimidine

The sulfapyrazoles, sulfanilamide itself and sulfanilic acid were not adsorbed within the experimental error (see below).

From the above experiments it appears that the order of sequence of increasing adsorption is the same as the order of sequence for increasing activity. Indeed a quantitative correlation between activity and adsorbability is evident from table I column c, where the adsorbed quantity,

Table I.

All concentrations are given in m. mol. per litre.

Column a: minimal concentration of the drug which is active i. e. reduces the number of viable bacteria below 10 per standard droplet i.e. 350 per ml in 24 hours (Exp. I).

Column b: activity coefficient with regard to sulfanilamide.

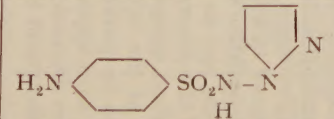
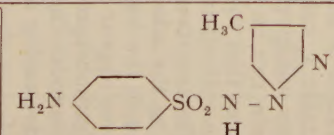
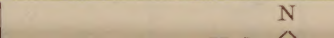
Column c: adsorbed quantity x/m in m. mol. per litre of bacteria (Exp. II) for $C_1$ = minimal active concentration.

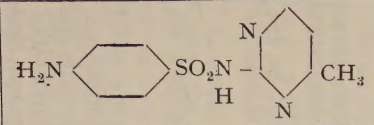
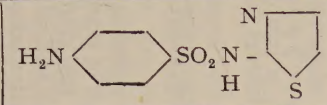
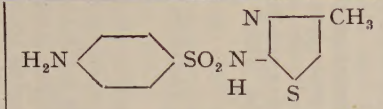
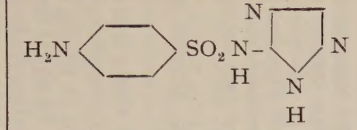
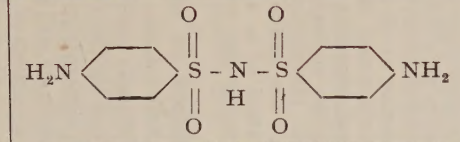
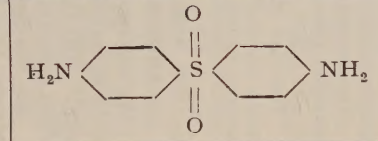
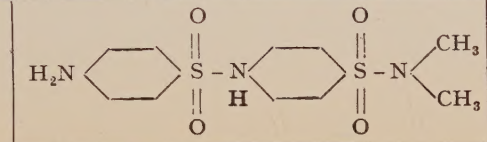
Column d: smallest concentration of p. aminobenzoic acid which is completely inhibiting the effect of the minimal active concentration of the drug (Exp. III).

Column e: a/d = ratio between active concentrations of p. aminobenzoic acid and sulfanilamide or related drugs.

Column f: b × e = 6/a × a/d = 6/d = constant.

□ = no adsorption within the experimental error.

| *Name* | *Formula* | *a* | *b* | *c* | *d* | *e* | *f* |
|---|---|---|---|---|---|---|---|
| Sulfanilic acid |  | 116 | 0.05 | □ | 0.005 | 23000 | ~ 1200 |
| Sulfanilamide |  | 6 | 1 | □ | 0.005 | 1200 | 1200 |
| 2-Sulfanilamido-pyrazol |  | 4 | 1.5 | □ | 0.005 | 800 | 1200 |
| 2-Sulfanilamido-4-methylpyrazol |  | 4 | 1.5 | □ | 0.005 | 800 | 1200 |
| |  | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2-Sulfanilamido-4-methylpyrimidine |  | 0.15 | 40 | 0.07 | 0.005 | 30 | 1200 |
| Sulfathiazol |  | 0.08 | 75 | 0.12 | 0.005 | 16 | 1200 |
| Sulfanilamido-methylthiazol (Methylsulfathiazol) |  | 0.07 | 85 | 0.15 | 0.005 | 14 | 1200 |
| Sulfatriazol |  | inactive > 4 | | □ | | | |
| Di-sulfanilamide |  | inactive > 4 | | □ | | | |
| Diamino-diphenyl-sulfon |  | 0.8 | 7.5 | 4 | 0.005 | 160 | 1200 |
| „Uliron" |  | inactive > 0.028 (saturated) | | strongly adsorbed | | | |

which corresponds with the concentration, which is the minimum active in the growth experiments, is given. It is evident that the adsorbed quantities at active concentrations are equal (about 0.1 m.mol.).

This probably holds also for sulfanilamide itself, but escapes experimental corroboration, as the adsorbed amount (about 0.1 m.mol.) with a 10 % suspension of bacteria, only reduces the outer concentration from 6 m.mol. to 5.99 m.mol. This difference is within the experimental error. Supposing an experimental error of about 2 %, any adsorbed quantity represented by a point to the right of the straight line A-A in fig. 1 falls within this error. The adsorption by sulfanilamide f.i. might be represented by any (straight) line to the right of A-A. So the dotted curves drawn for the sulfapyrazoles, sulfanilamide and sulfanilic acid in fig. 1 are purely hypothetical of course. They were drawn, however, on the surmisal that for the latter four drugs the adsorbed quantity at the active concentration would also be about 0.1 m.mol.

Sulfatriazol and disulfanilamide, which are inactive in our growth experiments are, as was to be expected, not adsorbed.

From this quantitative correlation between activity and adsorbability one might conclude that the better action of the studied sulfanilamide compounds in vitro is due to a better adsorption.

Only two out of the thirteen substances studied did not show the described correlation. Diamino-diphenylsulfon is as strongly adsorbed as p. aminobenzoic acid and is only 7.5 times as active as is sulfanilamide, whilst Uliron which is adsorbed about as strongly as sulfathiazol, at its saturated concentration is not active at all in our experiments with *B. coli* (though it is for streptococci in vitro and in vivo).

The structural formulae of these substances might explain this: an aspecific adsorption at inactive centres or some kind of sterical hindrance might affect the effectivity of adsorbed molecules in such a case.

The fact that the action of diamino-diphenylsulfon is inhibited by the same concentration of p. aminobenzoic acid, which inhibits sulfanilamide action, corroborates this view (see section C).

### C. The inhibition of the action of various sulfanilamides by p.aminobenzoic acid.

As sulfapyridine is thirty times more active than sulfanilamide, what is to be expected for the counteracting concentration of p.aminobenzoic acid? Will the concentration in which this inhibitor just counteracts the drug also decrease, or will it be equal for sulfanilamide and sulfapyridine?

As the better action of sulfapyridine is due to better adsorption; this means, that in one thirtieth of the concentration of the sulfanilamide, the sulfapyridine is able to occupy an equal number of centres of adsorption as does sulfanilamide.

It is reasonable to suppose, that for complete inhibition of the sulfanilamide or sulfapyridine effect an equal number of p.aminobenzoic acid molecules will have to be adsorbed at these centres. Such an adsorption will occur at a given p.aminobenzoic acid concentration and will be (practically) independent of the kind of sulfanilamide compound to be displaced.

So it may be expected that the counteracting concentration of p.aminobenzoic acid will be equal for all sulfanilamide compounds.

We studied this counteraction of p.aminobenzoic acid by adding various concentrations of this substance to media, inoculated with *B. coli*, which contained the minimal active concentration of the studied sulfanilamide compounds and by determining the minimal p.aminobenzoic acid concentration that re-established normal growth. Here again the viable count was used as a measure. In Exp. III some instances of these experiments are given (Fig. 4 and 5).

In table I, column d, the minimal inhibiting p. aminobenzoic acid concentrations are given. The concentration in which p. aminobenzoic acid counteracts appears to be equal for all the drugs studied.

In column e the ratio between the minimal active concentration of the sulfanilamide compound (column a) and the corresponding p. aminobenzoic acid concentration (column d) is calculated. For sulfanilamide this ratio is 1200, for sulfapyridine, which is 30 times as active the ratio is 40. The product of the activity quotient (column b) and the discussed ratio has to be constant of course ($30 \times 40 = 1200$). This shows that the ratio e has no vital meaning, but is dependent on the activity of the sulfanilamide compound. The really important fact is the constancy of the concentration in which p. aminobenzoic acid counteracts all the compounds studied.

From this fact it follows, that the number of adsorbed p.aminobenzoic acid molecules, which are necessary for the displacement of the sulfonamide, is the same for each compound. So it is evident, that the number of molecules displaced is also equal for each of the various compounds and this means, that at the different minimal active concentrations, the adsorbed quantities are equal.

From the experimental fact that the minimal concentration, in which p.aminobenzoic acid counteracts, is the same for all sulfonamides, we can independently again arrive at our former conclusion, that the different activity of these compounds is due to differences in adsorbability.

It would seem probable that the adsorbed quantity of p. aminobenzoic acid at the inhibiting concentration of 0.05 m.mol. should be also 0.1 m.mol. per litre of bacteria. Unfortunately this quantity can only be found by extrapolation. According to fig. 1 the adsorbed quantity of p.aminobenzoic acid per litre of bacteria should be 0.02 m. mol. Though this difference might be explained by the extrapolation and the experimental error, the chief difficulty lies in the fact that the adsorbed quantity corresponding with the inhibiting concentration of p.aminobenzoic acid cannot be read from this curve, which was determined in the absence of sulfanilamide compounds. Determinations of concurrent adsorption of p.aminobenzoic acid and sulfanilamide side by side will be necessary here.

## D. Experimental part.

Exp. I. Determination of the smallest concentration of sulfanilamide (or its derivatives) which is active.

*B. coli* was grown in a synthetic medium consisting of 1 g $K_2HPO_4$, 1 g $(NH_4)_2SO_4$ and 1 g Na pyruvate in 1000 ml tapwater. The medium was prepared in a more concentrated form and to 3 ml of this stock medium, 2 ml of solutions of the studied sulfanilamides in tapwater were added. With our usual technique (1) hourly determinations of the number of viable bacteria in these cultures were made. The growth curves,

derived from these data were plotted (f.i. fig. 2 and 3). In the ordinate the log of count is given per 28 mg of the culture. To obtain the log of count per ml log. 35 should be added. In fig. 2 and 3 instances of these experiments are given for sulfapyridine and methylsulfathiazol. For sulfanilamide the experiment has already been published (3).

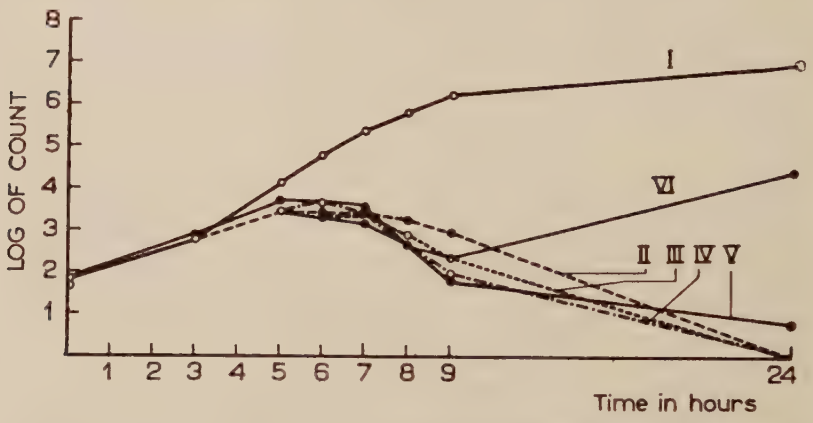



Fig. 2. The action of various concentrations of sulfapyridine on *B. coli*.

I Control. II, III, IV, V, VI with 2,1, 0.4, 0.2, 0.08 m.mol. sulfapyridine. The minima active concentration is 0.2 m.mol. (curve IV).

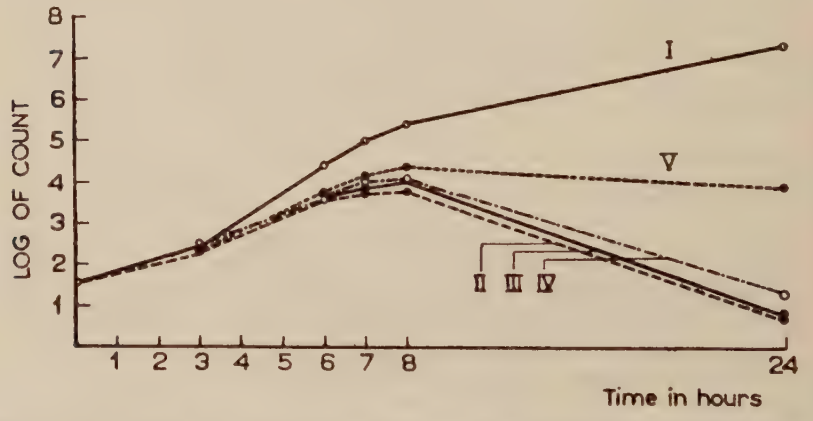



Fig. 3. The action of various concentrations of methylsulfathiazol on *B. coli*.

I Control. II, III, IV, V with 0.6, 0.2, 0.07, 0.03 m.mol. methylsulfathiazol. The minimal active concentration is 0.07 m.mol. (curve IV).

The smallest concentration, which within 24 hours reduced the number of viable bacteria below log.N = 1 was arbitrarily taken as a measure.

In most cases there was no difficulty in deciding upon the minimal active concentration. In some instances a culture with 0.1 % sulfanilamide was run simultaneously, thereby eliminating any possible differ-

ences between the various experiments, made at different times, with different batches of the medium etc. After finishing the determinations of the active concentrations of the studied compounds a further check was taken by testing the "active concentrations" of the six most important derivatives in one experiment. The obtained curves were essentially identical.

The minimal active concentrations for each drug are given in table I column a.

Exp. II. Determination of the adsorption of various sulfanilamide compounds by *B. coli*.

The method used in these experiments was described in full in no. VIII of this series (3).

*B. coli* was grown in glucose broth, the bacteria were centrifuged down, washed once with distilled water and resuspended in distilled water. The concentration of the suspension was estimated by centrifugation in capillary tubes and expressed as vol. %.

2 ml of a solution of sulfanilamide or one of the compounds were added to 2 ml bacterial suspension or to 2 ml distilled water. After centrifuging, the concentration of the drug in the supernate was determined (in duplicate) by diazotization and coupling to oxychinoline. The intensity of the orange-yellow colour of the diazo compound was estimated in a 3 cm cell in a Pulfrich Stuphometer with a filter S 50. The concentration of the drug in the supernate was derived from an extinction-concentration curve which was obtained with the control dilutions.

An instance of the determination of the adsorption isotherm $x/m = kc^{1/n}$ is given in table II. For p.aminobenzoic acid an analogous experiment was published in no. VIII of this series (3). All experiments were duplicated, generally with uniform results.

In table II an instance of an experiment is given for sulfapyridine with a 22 % suspension of bacteria. All concentrations are given in millimoles per litre; C = concentration in the control. $C_1$ = equilibrium concentration after adsorption (*i.e.* of the supernate).

The adsorbed quantity is obtained as a difference in concentration $C—C_1$. As the volume of the bacteria after dilution is 11 %, the adsorbed quantity $x/m = 11/100 \times (C—C_1)$ m.mol. per litre of bacteria. The concentration in the bacteria apart from the adsorbed quantity evidently is $C_1$

Table II

| C | $C_1$ | $C—C_1$ | x/m | log $C_1$ | log x/m |
|---|---|---|---|---|---|
| 0.400 | 0.382 | 0.018 | 0.162 | 0.58–1 | 0.20–1 |
| 0.200 | 0.192 | 0.008 | 0.072 | 0.28–1 | 0.86–2 |
| 0.100 | 0.096 | 0.004 | 0.036 | 0.98–2 | 0.56–2 |
| 0.050 | 0.048 | 0.002 | 0.018 | 0.68–2 | 0.26–2 |

We wish to draw attention to the fact, that for the curves V and VI and to a lesser degree also for curve IV (fig. 1), the adsorbed quantity is only just above the experimental error. And though the order of sequence of these three drugs was established beyond doubt, the absolute position of the curves should be regarded with some caution.

Exp. III. Determination of the concentration of p.aminobenzoic acid, which completely inhibits the minimal active concentration of a sulfanilamide compound.

*B. coli* was cultured in the synthetic medium already mentioned (Exp. I) and the influence was studied of various concentrations of p.aminobenzoic acid on the action of the sulfanilamide-compounds under discussion in their minimal active concentrations. The viable count,

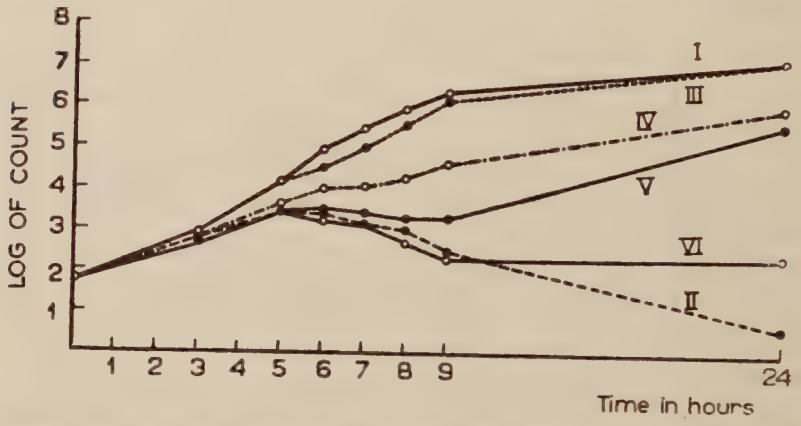



Fig. 4. The inhibition of sulfapyridine action by p. aminobenzoic acid (*B. coli*).

I Control. II with 0.2 m.mol. sulfapyridine. III, IV, V, VI with 0.2 m.mol. sulfapyridine and 0.005, 0.0017, 0.0005, 0.0001 m.mol. p. aminobenzoic acid respectively. Complete inhibition with 0.005 m.mol. p. aminobenzoic acid (curve III).

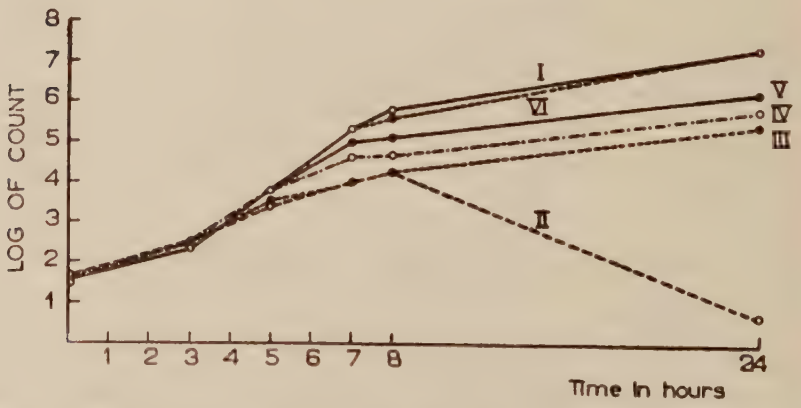



Fig. 5. The inhibition of 2-sulfanilamido-4-methylpyrimidine by p.aminobenzoic acid (*B. coli*).

I Control. II with 0.15 m.mol. 2-sulfanilamido-4-methylpyrimidine. III, IV, V, VI with 0.15 m.mol. 2-sulfanilamido-4-methylpyrimidine and 0.0001, 0.0005, 0.0017, 0.005 m.mol. p. aminobenzoic acid. Complete inhibition with 0.005 m.mol. p. aminobenzoic acid (curve VI).

made with our usual technique was used as a measure. Some instances of the resulting growth curves are given in the figs. 4 and 5 (for sulfapyridine and 2-sulfanilamido-4-methyl-pyrimidine respectively). For sulfanilamide an analogous experiment has already been published in no. VIII of this series (cf. fig. 4, p. 149 (3)). The inhibiting concentration of p.aminobenzoic acid derived from these growth curves is given in table I, column d.

p.Aminobenzoic acid in a concentration of 0.005 m.mol. per litre is able to re-establish growth completely in every case. In some cases (f.i. no. VIII of this series, fig. 4) the growth curve with 0.005 m.mol. p.aminobenzoic acid was not quite identical with the control.

Considering this and the experimental error of the determination of the inhibiting concentration it is possible that this concentration should be estimated slightly higher than 0.005 m.mol. It is quite evident, however, that the inhibiting concentrations were equal in all cases.

## Summary.

The better activity (in vitro) of various sulfanilamide compounds as compared with sulfanilamide itself is only quantitative, *i.e.*, an equal activity is obtained with lower concentrations. It is shown, that the activity of the studied drugs is so narrowly related to their adsorption in the bacteria (*B. coli*), that probably the varying activity of the studied compounds is due to differences in adsorbability. For different drugs the adsorbed amount was equal for concentrations with equal activity.

The concentration of p.aminobenzoic acid which re-establishes growth — in cultures containing the studied compounds in concentrations of equal activity — was equal in all cases. This fact corroborates the hypothesis, that activity and adsorption are correlated and shows, that the mechanism of action (in vitro) is the same in all cases.

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# VI-ANTIGEN IN *B. COLI* AND VI-AGGLUTININ

by

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The serological diagnostics of the *Salmonella* infections have been much improved owing to our knowledge of the antigenic structure of this bacteria, which has been laid down in the KAUFFMANN–WHITE scheme. For the differentiation of the *Salmonella* group many O- and H-antigens are used, as well as the Vi-antigen discovered by FELIX and PITT (10) with *S. typhi*. Besides in *S. typhi* this latter antigen is only found in *S. paratyphi* C (KAUFFMANN (14)), *S. ballerup* (KAUFFMANN and MØLLER (17)) and *B. coli* (KAUFFMANN (15)).

As Vi-antigen containing *S. typhi* are especially derived from chronic carriers of bacteria (CRAIGIE and BRANDON (4), WELCH and MICKLE (25), OGONUKI (20), FELIX (9), RAUSS (23) a.o.) various workers tried to trace these carriers by proving the presence of the Vi-agglutinin corresponding with the Vi-antigen in their bloodserum. According to information given by FELIX, ELIOT (6), BHATNAGER (2), PIJPER and CROCKER (21), GIOVANARDI (12), FAARUP (7) and RAMMLER (22), this method leads to very good results.

DAVIS (5) examined the serum of 656 natives from South-Rhodesia and he found the Vi-agglutinin in 49 cases (= 7.5 %) with a titer from 1 : 5 to 1 : 12.5. FAARUP found the Vi-agglutinin in 15 out of 190 normal sera, *i.e.*, 8 %, with titers varying from 1 : 10 to 1 : 50, whilst RAMMLER succeeded in showing the presence of the latter in 8 out of 188 normal sera (4 %) with a titer of 1 : 5.

Investigating the presence of *Salmonella* antigens in *B. coli* we (1) found that the Vi-antigen occurred in 7—8 % of the cases wherein *B. coli* had been derived from the intestines of healthy persons. In connection with the problem of the genesis of the natural agglutinins we thought it of interest to investigate, whether there could be found any connection between the Vi-antigen in *B. coli* from the intestines and the Vi-agglutinin in the blood of healthy individuals; in this way we meant to answer the question whether this natural Vi-agglutinin is the consequence of an infection by the pathogene *S. typhi*, or that it might be caused by the light attacking action that the commensal *B. coli* can maintain within the host. There are, as known, two theories about the origin of the natural agglutinins; the first regards them as a token of physiological ripening (serogenesis of H. and L. HIRSCHFELD) and the second attributes them to the presence of an other bacterium with similar antigens (for a good review of this problem we refer to REURINK (24)).

For this purpose we investigated 600 sera having been sent to us for

examination by means of the WASSERMANN-reaction and proved negative for the latter as to the occurrence of the Vi-agglutinin.

As ficker we used a suspension in formaldehyd of the Vi-I strain of BHATNAGER (11). This strain exclusively possesses the Vi-antigen, no O- or H-antigen (the use of a strain of *B. coli* with Vi-antigen without other *Salmonella* antigens gave about the same results). The agglutination was read in the dilutions 1 : 5, 1 : 10, 1 : 20, 1 : 40 etc. after the sera had been kept for two hours at 37° C. and at room temperature during 20 hours.

54 out of these 600 sera proved to contain Vi-agglutinin, *viz.*, 30 with a titer of 1 : 5, 19 with a titer of 1 : 10, 4 with a titer of 1 : 20 and 1 with a titer of 1 : 40. In these cases we were supplied with faeces from the individuals concerned in order to be able to investigate whether these samples contained *B. coli* with Vi-antigen. Moreover, as a control, we also examined faeces originating from healthy persons, who had no Vi-agglutinin in their serum. In some cases, in which the Vi-agglutinin was found in the serum, an infection with typhoid or paratyphoid appeared to have occurred in the anamnesis or an immunization against such an infection. Of course such cases were left out of account.

For comparison with *B. coli* from the faeces there remained 32 sera with Vi-agglutinin and 26 sera without Vi-agglutinin. We arrived at the following results:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Vi-agglutinin | in | serum | +, | Vi-antigen | in | *B. coli* | + : 14 × |
| ,, | ,, | ,, | +, | ,, | ,, | ,, | — : 18 × |
| ,, | ,, | ,, | —, | ,, | ,, | ,, | + : 10 × |
| ,, | ,, | ,, | —, | ,, | ,, | ,, | — : 16 × |

Apparently we have not succeeded in proving a connection between the Vi-agglutinin in the serum and the Vi-antigen in *B. coli* from the intestines, for this antigen was found about as many times together with the agglutinin as in its absence and conversely.

When these experiments were effected, the L-antigen of *B. coli* had not yet been described by KAUFFMANN (16). Whenever this antigen is found, *B. coli* shows the phenomenon of O-inagglutinability, but it is not stated, whether also the Vi-agglutinability disappears. Yet this L-antigen can have had little if any influence on the outcome of our experiments, as it is principally found by means of *B. coli* isolated from urine during infections of the urinary ducts, from pus, gall, blood etc., and seems to occur seldom in *B. coli* isolated from sound intestines (KAUFFMANN and PERCH (18)). Nevertheless the possibility of a connection between the Vi-agglutinin and the Vi-antigen of *B. coli* is not yet wholly excluded by the result of our experiments, for examinations made by MIKKELSEN (19), by KAUFFMANN and PERCH (18), by BOIVIN, CORRE and LEHOULT (3), and by ourselves (1), have made it known, that in the intestines different strains of *B. coli* may occur together and that some strains may disappear, which may reappear later on.

As we were only in a position to examine for each case a single sample of faeces, we could only make a so-called snapshot, which needs not necessarily be a true picture of the real situation in the intestinal canal and so the possibility exists that strains with Vi-antigen have been present without having been demonstrated by us.

As regards the absence of the Vi-agglutinin in those cases in which *B. coli* with Vi-antigen were found, we mean to suggest that not every

individual needs to react upon the presence of an antigen by forming an agglutinin and moreover it is possible, that the antigen found had not yet lead to the formation of an agglutinin, the presence in the body having not lasted long enough for this.

So further experiments will be necessary to solve this problem.

At first we had the intention to examine the problem also experimentally in the same way as INGALLS (13) did, by feeding young rabbits with *B. coli* containing Vi-antigen, and by examining whether the Vi-agglutinin would appear in the serum after some lapse of time. Present conditions, however, have prevented us to accomplish these experiments with animals, but we hope to be in a position to do so as yet in due course.

## Summary.

The Vi-agglutinin in normal serum occurs in 4—8 %, the Vi-antigen in *B. coli* out of faeces of healthy persons in 7—8 % of the cases. The authors could not, however, prove a connection between these two phenomena.

The authors present some reasons why such a connection need not to be considered as absolutely impossible after all.

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# THE CLASSIFICATION OF THE PLAGUE-BACILLUS

by

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In the fifth edition of BERGEY's Manual of Determinative Bacteriology (1939) the genus *Pasteurella* — together with the genera *Brucella* and *Hemophilus* — has been placed in the family *Parvobacteriaceae*, a classification recently proposed by RAHN (4).

Consequently the plague-bacillus (*Pasteurella pestis*) and the bacillus of pseudotuberculosis rodentium (*Pasteurella rodentium*), although both of moderate size, are automatically included in a family which is characterised, as has been postulated by its author, by the exceptionally small size of its members.

It is therefore apparent that the classification of the plague-bacillus and of the rodentium bacillus, which also previously was far from satisfactory, urgently needs revision.

The relation between the plague-bacillus and the type-species *Pasteurella avicida* is by no means close. They differ not only by size, but by their biochemical properties, antigenic structure and the character of their agar colonies as well (See Table).

The main reason that they have been grouped in the same genus seems to be that in stained preparations the ends of the rods take the dye more markedly than the central part, especially in bacteria derived directly from the body. However it must be recognised that occasionally this character is also present in other genera and that even in this bipolar staining the plague-bacillus differs from the typical *Pasteurella*. In the latter the bipolarity is far more regular than in the former; very often smears from infected material contain plague-bacilli which did not take the dye exactly at the ends, but more or less obliquely or even at one side only (securety-pin).

RAHN (4) himself recognises the exceptional position of the „large" plague-bacillus among the *Parvobacteriaceae*, stating that „the relationship of this group must be considered only tentative".

These are the motives for my proposition to classify the plague-bacillus, together with the nearly related rodentium bacillus, in a new genus: *Yersinia* [1]) (*Y. pestis*, *Y. rodentium*) and to place this genus, with other unclassifiable genera into the family of the *Bacteriaceae*.

[1]) A. J. E. YERSIN (1863–1943) discovered the plague-bacillus in 1894.

Table

| | Plague-bacillus (*Yersinia pestis*) | *Pasteurella avicida* |
|---|---|---|
| Average length | 1.5—1.8 μ (ALBRECHT and GHON cf. (2)) | 0.15—0.25 μ (HUTYRA cf. (3)) |
| Growth in plain commercial meat extract agar | abundant | scanty |
| Colony on agar | raised opaque yellow centre and flat, clear periphery with crenellated margins | smooth, translucent, even-edged |
| Growth on potato | + | — |
| Growth in yeast-water (cf. (1)) | + | — |
| Growth in the presence of bile salts | + | — |
| Rhamnose | + | — |
| Saccharose | — | + |
| Sorbitol | — | + |
| Indol | — | + |
| $H_2S$ | — | + |
| Antigenic structure | No cross-agglutination | |
| Plague-phage (cf. (1)) | + | — |

I am not quite sure whether the name *Yersinia* has ever been proposed; I did not succeed, however, to retrace it in literature.

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(From the Department for Bacteriology and Experimental Pathology of the Institute of Preventive Medicine and from the Pathological Laboratory of the University at Leiden).

# A PHENOMENON RESEMBLING ANAPHYLACTIC SHOCK AFTER TREATMENT WITH SULPHAPYRIDINE

by

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In two earlier communications (4,6) we have offered arguments for the conception that the fatal course of cases of pneumonia treated with sulphapyridine might have been caused by general anaphylactic reaction and that the necroses in lung tissue we could note in some of such cases might be due to a local anaphylactic reaction. This conception is based on the results we have arrived at in experiments on rabbits with filtrates of inflammated lungs of rabbit. In fact a filtrate prepared out of inflammated lungs of rabbit having been treated with sulphapyridine could induce an anaphylactic shock in a notably smaller amount than when no sulphapyridine had been administered. So in rabbits, which had outlived a pneumonia, sulphapyridine lung filtrate could give rise to shock in an amount at least ten times smaller than the amount needed when no treatment with sulphapyridine had taken place. So we could conceive that patients suffering from pneumonia might also be sensibilized to one or more antigens present in the inflammated lungs and that the treatment with sulphapyridine might further the occurrence of anaphylaxis. In our formerly described experiments on animals we had not succeeded in inducing a chemospecific anaphylaxis with sulphapyridine. WERTH (5), however, has proved that sulphapyridine without the addition of protein can exercise anaphylactic action on guinea pigs. A guinea pig, intraperitoneally sensibilized with sulphapyridine, shows after three weeks a positive DALE test.

Some further facts induced us to take up again the investigation regarding an existence of chemospecific anaphylaxis to sulphapyridine. These facts concern two patients who had died at the Surgical Clinic at Leiden (Prof. SUERMONDT), respectively nine and ten days after a succesful surgical intervention.

The first patient, a man of 44 years, had been suffering for a few years from gastric ulcer. Notwithstanding a conservative treatment his complaints

had not decreased. Blood had been repeatedly detected in the stools. Therefore an operation (gastric resection) was performed.

The second patient, a man of 42 years, had been admitted on account of a perforation of the stomach. In the previous year he had shown symptoms of gastric ulcer. Besides this he had always been in good health.

After the operation sulphapyridine had been administered to both patients (1—4 g intramuscularly) during respectively eight and five days in view of the possibility of postoperative pneumonia. Two or three days after the operation the general condition of both patients was excellent. Then both developed fever. A few days later the first patient showed cyanosis. During the following five days the body temperature kept on increasing. Then he collapsed and died on the same day. The second patient expectorated a purulent sputum at the seventh day after the operation. Two days later also his general condition deteriorated rather suddenly and he died on the next day. The administration of sulphapyridine had been brought to a close three days before his death, whilst the first patient had received this drug up till the day before his death.

Post mortem examination revealed in both cases hemorrhagic serous cellular bronchopneumonia in the caudal parts of the lungs. Hyperaemia of the lungs and of the abdominal viscera occurred. The heart did not show any particular abnormality. In the operation region no definite alterations could be noted. The peritoneum was smooth and glossy.

The fact that on the one hand before the operation both patients had been in good condition and on the other hand that the operation in both cases had been very successful, which latter fact was confirmed by the obduction, made us doubt whether the pathological findings: serous cellular bronchopneumonia, could actually account for the death. In this connection the surgeon (H. G. Ruhaak) put in the question whether the prophylactic administration of sulphapyridine might not in these cases have affected the course of the disease unfavourably.

As in our opinion in pneumonia anaphylaxis can be furthered by the administration of sulphapyridine the question arose, whether in the cases at hand the reverse might not have taken place. In other words: might this be a case of sensibilization by sulphapyridine and is it possible that the antigens which are formed in the course of pneumonia appearing after the sensibilization, might have furthered the arising of an anaphylactic reaction? In order to be able to answer this question, we tried again to induce a chemospecific anaphylaxis in guinea pigs with sulphapyridine. In this experiment the arrangement such as it was followed in our former experiments was somewhat altered. At the same time we investigated whether the filtrate of inflammated human lung tissue might exercise a shock on guinea pigs having been sensibilized with sulphapyridine and whether the filtrate might further the arising of chemospecific anaphylaxis.

To man sulphapyridine is administered per os as well as parenterally during several days at a stretch. According to the present-day conceptions about the arising of anaphylaxis an anaphylactic reaction might be expected only when the sensibilization had been induced by a small dose of anaphylactogen administered parenterally, followed after at least ten days by a re-injection of the same

anaphylactogen directly into the bloodvessel in a dose several times the strenght of the initial one. Repeated administrations such as were performed on our patients would render a desensibilization rather more probable than a sensibilization. Moreover the sulphapyridine had been injected during eight and five days; so the incubation period had been rather short for an anaphylactic reaction. We have tried, however, to sensibilize guinea pigs with sulphapyridine by one or more intramuscular injections of 50 mg (on successive days) or by doses per os of 250 and 500 mg. The anaphylactic injection was administered intracardially with 100 mg of sulphapyridine at various times after the first administration.

Table I

| Guinea pig | sensibilization | | anaphylactic injection | | shock |
|---|---|---|---|---|---|
| | dose of sulphapyridine | mode of administration | dose of sulphapyridine | days after the first injection | |
| 159 | 1 × 50 mg | intramuscular | 100 mg | 1 | — |
| 160 | 2 × 50 ,, | ,, | ,, | 3 | — |
| 162 | 3 × 50 ,, | ,, | ,, | 4 | — |
| 168 | 1 × 50 ,, | ,, | ,, | 4 | — |
| 161 | 4 × 50 ,, | ,, | ,, | 5 | + |
| 169 | 2 × 50 ,, | ,, | ,, | 5 | + |
| 170 | 2 × 50 ,, | ,, | ,, | 7 | + |
| 164 | 5 × 50 ,, | ,, | ,, | 8 | — |
| 163 | 5 × 50 ,, | ,, | ,, | 10 | — |
| 165 | 6 × 50 ,, | ,, | ,, | 10 | + |
| 171 | 2 × 50 ,, | ,, | ,, | 10 | + |
| 166 | 7 × 50 ,, | ,, | ,, | 11 | — |
| 167 | 7 × 50 ,, | ,, | ,, | 12 | — |
| 172 | 2 × 50 ,, | ,, | ,, | 12 | + |
| 153 | 5 × 50 ,, | ,, | ,, | 14 | + |
| 154 | 5 × 50 ,, | ,, | ,, | 14 | + |
| 264 | 1 × 500 ,, | per os | ,, | 6 and 14 | — |
| 286 | 4 × 250 ,, | ,, | ,, | 8 | — |
| 275 | 1 × 500 ,, | ,, | ,, | 13 | + |
| 265 | 1 × 250 ,, | ,, | ,, | 14 | + |
| 280 | 4 × 250 ,, | ,, | ,, | 14 | + |
| 283 | 4 × 250 ,, | ,, | ,, | 14 | + |
| 276 | 1 × 250 ,, | ,, | ,, | 16 | — |
| 288 | 1 × 500 ,, | ,, | ,, | 21 | — |
| 289 | 1 × 250 ,, | ,, | ,, | 21 | — |
| 291 | 1 × 500 ,, | ,, | ,, | 21 | — |

As is shown in table I sensibilization of guinea pigs with sulphapyridine would actually be possible per os as well as intramuscularly by repeated administration. The intracardial anaphylactic injection gives rise to the symptoms of shock in 40—50% of the animals, none of which cases, however, are fatal. A deadly shock, however, can be provoked by means of a re-injection with a mixture of sulphapyridine and lung filtrate.

Table II

| Guinea pig | sensibilization | | anaphylactic injection | | days after the first injection | shock |
|---|---|---|---|---|---|---|
| | dose of sulphapyridine | mode of administration | dose of sulphapyridine | dose of lung filtrate | | |
| 146 | 40 mg | intramusc. | 200 mg | 1 cc | 14 | ++ |
| 149 | 20 ,, | ,, | 200 ,, | 1 ,, | 14 | — |
| 152 | 40 ,, | ,, | 100 ,, | 1 ,, | 14 | ++ |
| 155 | 5×50 ,, | ,, | 100 ,, | 1 ,, | 14 | + |
| 153 | 5×50 ,, | ,, | 100 ,, | 1 ,, | 14 | + |
| 150 | 40 ,, | ,, | | 2 ,, | 14 | — |
| 151 | 60 ,, | ,, | | 3 ,, | 14 | — |
| 157 | 5×50 ,, | ,, | | 2 ,, | 14 | — |
| 158 | 5×50 ,, | ,, | | 2 ,, | 14 | — |
| 281 | 4×250 ,, | per os | | 2 ,, | 14 | — |

++ deadly shock.
+ non-deadly shock.

The lung filtrate has been prepared out of inflammated human lung tissue. For this purpose this matter is cut to pieces and ground finely; it is subsequently suspended in 10 parts of a physiological saline solution (0.9% NaCl), shaken for two hours and finally filtered of through a double gauze and through a Seitz E.K. filter.

The intracardial injection of the lung filtrate on guinea pigs sensibilized with sulphapyridine and the injection of sulphapyridine lung filtrate in non-sensibilized guinea pigs does not give rise to any reaction.

The actual possibility to sensibilize a guinea pig per os seems to disagree with the conception about the arising of protein anaphylaxis. Though in exceptional cases it is possible for a protein to pass the intestinal wall, the proteins are generally broken down in the intestinal tract, thus losing their antigenic character and sensibilizing action. Sulphapyridine however is not a protein and so it can pass the intestinal wall in an unchanged condition. According to KIMMIG and WESELMANN (3) the sulphanilamides are haptens, which only attain an antigenic character by their linkage to serum protein. We can imagine that by these means sulphapyridine notwithstanding the peroral administration may appear in the blood and may exert an antigenic action after its linkage to the serum or organ proteins and may sensibilize the organism in that way. Contrary to the anaphylactic shock in such experiments shock symptoms can be induced as early as five days after the initial injection, whilst a desensibilization by the repeated intramuscular and peroral doses has not been induced. Moreover Table I shows that the sensibilized animals remain sensitive to the shocklike symptoms during a merely short period, for this phenomenon only appears in guinea pigs that have been sensibilized 5 or 14 days earlier. Whereas after a lapse of 21 days

the phenomenon can no longer be induced. The protein anaphylaxis, on the other hand, remains for life. The anaphylactic reaction is regarded as an antigenic-antibody reaction. It cannot be conceived that in merely 5 days antibodies would have been formed. So we are of the opinion that these arguments can be advanced against the conception that the symptom described by us might be a mere anaphylactic reaction.

How can we account for it, however, if not in this way? The doses of the sulphapyridine injected are not toxic, nor is the toxic dose reached by cumulation. If we inject a guinea pig intracardially with 400 mg of sulphapyridine the animal shows, not immediately but after a short lapse of time, convulsions which may last for one hour and longer. The shock symptom, however, only appears in sensibilized guinea pigs immediately after the intracardial re-injection. Clinically it cannot be distinguished from the anaphylactic shock and it lasts for five minutes at most. At autopsy the guinea pigs (after the injection with sulphapyridine lung filtrate, Table II) show hyperaemia of the abdominal organs, emphysematous changes of the lungs and hemorrhagiae are found under the pleura pulmonalis. Like symptoms, among which the hemorrhagic reaction, could also be noted in the patients. A similar reaction is seen in the SANARELLI–SHWARTZMAN phenomenon. As it is known a rabbit will die with the symptoms of anaphylactic shock after the intravenous injection of colifiltrate, provided that the animal has been injected 24 hours earlier with a subletal dose of cholera vibrios (phenomenon of SANARELLI) and that a hemorrhagic necrotic reaction will appear on a rabbit after the intravenous injection of a filtrate of *B. coli*, *B. typhosus* or other bacteria, provided that such a filtrate has been injected intracutaneously 24 hours earlier (phenomenon of SHWARTZMAN).

With chemical substances as well a phenomenon can be excited which shows some analogy to the former phenomena, *viz.*, the phenomenon of GLAUBACH (2). Here a deadly shock can be induced in rats by means of the subcutaneous injection of a harmless dose of papaverine 2—3 hours after the peroral administration of a harmless dose of sulphapyridine (DUREL and RATNER (1)).

Though the shock phenomenon observed by us can appear no earlier than after five days and the phenomena of SANARELLI–SHWARTZMAN and of GLAUBACH appear already after respectively 24 and 2 hours, it is yet possible that they may be of a same nature. In each of these phenomena after a preceding sensibilization a shock will appear which cannot be distinguished from an anaphylactic shock. Whilst the anaphylactic reaction, however, is strictly specific, *i.e.*, only appears when the same substance is used in the reinjection as in the initial sensibilizing injection, the phenomena of SANARELLI–SHWARTZMAN and of GLAUBACH are induced by a substance other than the initial one. The phenomenon described by us is essentially specific to sulphapyridine but it is intensified moreover by an unspecific component, *viz.*, the lung filtrate. The

results of our experiments might point to the possibility of sulphapyridine exerting a sensibilizing action on man as well and we can imagine that in case a pneumonia develops notwithstanding the prophylactic treatment with sulphapyridine shock phenomena are more likely to appear than without the occurrence of pneumonia.

If this is true death can be only accounted for by shock. This would agree with the fact that in the two fatal cases described the pneumonic alterations found cannot sufficiently account for the death. Already at the beginning of pneumonia, however, strange proteins (organ- and species-specific ones) which can pass into the blood, are formed in the lungs and these may be put on a level with the lung filtrate used in the experiments on guinea pigs.

## Summary.

Two patients on which a successful operation of the stomach had been performed developed fever some days after the operation, notwithstanding a prophylactic treatment with sulphapyridine and both of them died rather suddenly respectively 9 and 10 days after the operation. On obduction in both cases hemorrhagic serous-cellular bronchopneumonia were found in the caudal parts of the lungs, all the organs were very hyperaemious and the heart did not show any alteration. To account for the fatal course the possibility of chemospecific anaphylaxis to sulphapyridine has been considered. We succeeded in inducing in guinea pigs by means of sulphapyridine a shock, which, however, did not result in death. Such a shock could be induced as early as five days after sensibilization. The adding of the filtrate of inflammated lung tissue resulted in a deadly shock. In this connection the surmisal was made that also in the patients a sensibilization by sulphapyridine had occurred and that the pneumonia, which as such could not sufficiently account for the death, has furthered the arising of shock. We have pointed to the various facts which disagree with the identification of the phenomenon observed with an anaphylactic shock. We mention, however, that there is a certain agreement with the phenomenon of SANARELLI–SHARTZMAN and that of GLAUBACH.

## Literature.

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# ÉPIDÉMIE SURVENUE PARMI DES SOURIS BLANCHES, À LA SUITE D'UNE INFECTION PAR LE *CORYNEBACTERIUM PSEUDOTUBERCULOSIS MURIUM*

par

**M. F. POLAK**

(Reçu le 30 Septembre 1943).

Parmi les épidémies qui peuvent survenir au laboratoire chez les souris blanches, quelques-unes sont caractérisées par une formation de tubercules en divers organes et comprises par le nom de „pseudotuberculose". Pour autant qu'elles appartiennent au domaine bactériologique, les espèces suivantes sont principalement indiquées comme agents de cette pseudotuberculose: *Bact. pseudotuberculosis rodentium* (*Pasteurella pseudotubercolosis*), différentes *Salmonellae* et le *Corynebacterium pseudotuberculosis murium*. REZEK et LAUDA (4) communiquent, dans leur discussion de la pseudotuberculose du foie des animaux de laboratoire, caractérisée par des nécroses en foyers à réaction inflammatoire, que *Corynebacterium pseudotuberculosis murium* ne provoquerait jamais d'anomalies dans cet organe. Ceci n'est pas en complet accord avec les données bibliographiques; or, ayant pu observer parmi les souris blanches de l'„Instituut voor Tropische Hygiëne" une épidémie provoquée par cette bactérie et dans laquelle, seul, le foie était atteint, nous donnons ici la description de nos observations.

Au cours de l'an dernier, lors de nos expériences effectuées sur des souris blanches pour des recherches sur la fièvre jaune, il surgit des difficultés par suite d'une infection introduite par deux fois dans notre matérial avec un envoi de souris livrées par un même fournisseur. Peu après l'arrivée des animaux au laboratoire, il survint des cas de morts „spontanées" enlevant plusieurs souris par jour, de sorte que quelques semaines plus tard, il restait fort peu d'animaux d'un groupe de 50 souris. Le syndrome clinique était peu caractéristique: les animaux perdaient leur activité, leur pelage devenait hirsute, ils se tenaient immobiles, le dos en boule et leur défécation était fréquemment de consistance liquide. L'autopsie, toutefois, révéla que le foie présentait toujours un aspect caractéristique: il était parsemé de petits foyers d'un blanc grisâtre,

de 2 mm de diamètre au plus, pas très ronds et mal délimités d'avec le tissu normal environnant. La rate était grosse, mais par ailleurs normale. Des cultures, faites à l'aide de ces foies, donnèrent un micro-organisme présentant une parenté très étroite avec le *„Bacillus" pseudotubercolosis murium* que KUTSCHER (3) isola en 1894 de poumons de souris infectées et que l'on compte maintenant parmi les diphtéroïdes sous le nom de *Corynebacterium pseudotuberculosis murium.* Il a été fait un examen microscopique du foie de l'une de nos souris: les petits foyers consistaient en infiltrations de polynucléaires avec quelques lymphocytes et, souvent, avec nécrose centrale du tissu hépatique; les limites des cellules étaient vagues, les noyaux pyknotiques et fragmentés. Le Gram révéla que les infiltrations étaient pleins de bactéries: bâtonnets minces et parfois un peu arqués, positifs au Gram et de longueur variée, atteignant au maximum 3 $\mu$. Ils étaient irrégulièrement colorés et montraient parfois une coloration des granules et des pôles; ils ressemblaient fortement à des bactéries de diphtérie. Les micro-organismes se bornaient pour ainsi dire au tissu anormal, bien qu'il y eût parfois encore quelques groupes dans le territoire avoisinant.

L'ensemencement de foyers de foie sur le sang gélosé nous donna plusieurs fois une culture pure de bactéries de ce genre. Or, maintenant, le Gram les montrait plus courtes (1—2 $\mu$) et de forme plus lourde souvent amassées en formation V. Colorées selon Neisser, elles montraient, quelquefois rarement et quelquefois en abondance, une coloration polaire, qui, d'ailleurs, n'impliquait pas toujours les deux pôles. Les grains ne faisaient pour ainsi dire jamais saillie à l'extérieur du bâtonnet et ils étaient quelquefois très gros.

Sur la gélose, les bactéries avaient formé, en 3 fois 24 heures (par 37° C.), de petites colonies rondes, d'un blanc jaunâtre, quelque peu transparantes, à surface convexe et finement granulée avec un bord très net. En 6 fois 24 heures, leur diamètre avait atteint 0.5 à 1 mm. Sur le sang gélosé et dans le sérum coagulé, la croissance avait été beaucoup plus rapide. La gélatine en piqûre ne montrait aucune croissance après 24 heures; après 72 heures et plus, on y voyait un trait d'ensemencement grisâtre non branché. Le bouillon était trouble après 1 jour, avec un léger dépôt tourbillonnant rapidement au secouage; nous n'avons pas observé de formation cristalline. Les micro-organismes étaient toujours immobiles.

Le comportement biochimique était celui-ci: pas de formation d'indol, pas de modification du lait tournesolé et du milieu d'Oldekopp, fermentation de glucose, maltose et saccharose causant une acidification sans gaz, tandis que lactose, mannitol, glycérol et adonitol ne sont pas détruits. La gélatine n'est pas liquéfiée, le lait ne coagule pas; il n'y a ni hémolyse, ni hémodigestion.

Ces caractéristiques établies, il nous semble pouvoir dire que la bactérie que nous avons isolée est un diphtéroïde, identique ou

étroitement apparenté au *Corynebacterium pseudotuberculosis murium* que KUTSCHER décrivit le premier. D'autres auteurs ont, eux-aussi, isolé des bactéries de ce genre chez des souris ou des rats (REED (5), BONGERT (1), GUNDEL, GYÖRGY et PAGEL (2)); ils mentionnent de petites différences dans la morphologie et le mode de croissance, le syndrome provoqué chez les souris n'est pas, lui non plus, toujours absolument le même. KUTSCHER vit une seule fois, chez l'une de ses souris d'expérience, de petits foyers hépatiques; l'inoculation sous-cutanée de sa bactérie n'amenait point la mort de ses souris. Par contre, BONGERT observa plusieurs fois des anomalies dans le foie et, de plus, dans les reins et la rate; il vit souvent aussi du gonflement des glandes lymphatiques. L'inoculation sous-cutanée était mortelle. REED n'a jamais observé de foyers hépatiques lors de ses expériences d'infection. Toutefois, lors d'infection spontanée, la localisation pulmonaire est très générale et le poumon présente de petits tubercules à centre caséeux. Ceci, pourtant, n'a jamais été le cas dans nos expériences; les petits foyers hépatiques étaient les seules anomalies visibles.

Afin de prouver que le corynebacterium isolé était l'agent de l'infection attaquant nos souris, nous avons pratiqué des expériences d'infection chez des souris blanches, saines, d'autre provenance.

L'injection sous-cutanée d'un 0.5 cc de suspension de bactéries (1/20 du tube de sérum coagulé incliné) provoqua la mort de 3 souris en, resp. 7, 11, et 14 jours. Il y avait, à la place de l'injection, une grande infiltration déjà plus ou moins ramollie. Le pus donna une culture pure de *Corynebacterium pseudotuberculosis murium.*

L'inoculation intrapéritonéale amenait la mort de la souris dans les 3 à 12 jours (dépendant, entre autre, de la dose). On découvrit, dans tout l'abdomen des animaux morts en peu de temps, une couche fibrineuse sur le péritoine viscéral et pariétal. Il se développait chez ceux ayant vécu plus longtemps de petits foyers hépatiques. Nous n'avons pas observé d'hypertrophie des glandes lymphatiques du mésentère. Le corynebactérium injecté a toujours pu être cultivé, à l'état pur, à l'aide du foie.

Du moment que nous supposions que l'infection naturelle se produit par la bouche, nous avons tenté d'infecter un certain nombre de souris au moyen des aliments et traité, à cet effet, 7 animaux. L'un mourut après 1 jour, un second survécut et fut sacrifié un mois après; il ne montrait aucune anomalie. Parmi les 5 autres, 3 moururent en, resp., 9, 13 et 14 jours, présentant des anomalies pulmonaires et 2, en, resp. 9 et 22 jours, présentant des anomalies pulmonaires et les petits foyers hépatiques en question. Peu avant leur mort, tous ces animaux avaient une dyspnée manifeste. Les symptômes pulmonaires nous surprirent; les poumons étaient remplis de tubercules blancs-roses, assez nettement délimités du tissu pulmonaire hyperémique, mais

normal par ailleurs; leur forme était irrégulière et leur diamètre mesurait 3—4 mm environ. Les tubercules étaient nettement proéminents à l'extérieur de la surface du poumon; la plèvre était adhérente, mais toujours sans exsudat. Les glandes lymphatiques de la poitrine et du cou n'étaient pas hypertrophiées. Nous avons cultivé plusieurs fois, à l'état pur, le *Corynebacterium pseudotuberculosis murium* à l'aide des foyers pulmonaires.

Ces tubercules des poumons concordent avec les anomalies que, d'après la bibliographie, on trouve régulièrement lors de l'infection naturelle par la bactérie de KUTSCHER; mais, dans notre cas, ils ne se sont manifestés qu'après une infection artificielle, dont le but était d'imiter l'infection naturelle. Il est possible que, lors de contamination orale moins massive en soi que celle appliquée dans nos expériences, les foyers hépatiques eussent été les seuls symptômes.

KUTSCHER n'a point réussi dans ses expériences à infecter des souris par la bouche; la vaporisation régulièrement pratiquée des suspensions de bactéries a toutefois provoqué les anomalies pulmonaires caractéristiques. TOPLEY et WILSON (6) n'ont observé les anomalies pulmonaires que de temps à autre, après l'infection artificielle, et en bien moindre mesure que lors d'infection naturelle. Quant à nous, c'est justement dans ce dernier cas que nous n'avons jamais vu de lésions pulmonaires, tandis que la contamination artificielle orale les provoquait.

Nous avons examiné plusieurs fois, au microscope, le foie et les poumons de souris infectées artificiellement. Le foie montrait des anomalies concordantes avec celles citées plus haut et il y avait, dans les poumons, des foyers étendus de pneumonie purulente; les alvéoles présentaient une forte infiltration de polynucléaires, tandis qu'on voyait en même temps des gros amas de bactéries ressemblant nettement à des corynebactériums, tant avec le Gram qu'avec le bleu de méthylène de Loeffler. D'autres parties du poumon montraient de grands hématomes.

L'injection intrapéritonéale de filtrat de cultures faites dans du bouillon n'amenait pas la mort des souris; à l'encontre d'autres auteurs, nous n'avons donc aucune raison d'admettre ici la formation d'une exotoxine. Mentionnons ensuite que l'ensemencement de sang cardiaque de souris infectées a eu régulièrement un cours négatif.

Nous pensons pouvoir conclure, en résumé, que le corynebactérium que nous avons isolé, est l'agent de l'épidémie constatée chez nos souris blanches. Cette bactérie appartient à un groupe prochement apparenté de diphtéroïdes dont KUTSCHER fut le premier à décrire un réprésentant. Ces organismes provoquent, chez la souris, des foyers d'infiltration nécrotiques, dont la dispersion dans l'organisme varie cependant.

La lutte contre la maladie comprend peut-être, outre la prise de mesures générales (isolation, sacrifice des animaux malades

et, éventuellement, des animaux suspects), une modification de l'alimentation. GUNDEL et ses collaborateurs communiquent que leurs rats n'eurent une pneumonie que s'ils souffraient d'une déficience en vitamine H. Cette substance a été active aussi thérapeutiquement.

## Résumé.

Une épidémie ayant atteint des souris blanches de laboratoire, a présenté le syndrome d'une pseudotuberculose du foie. Les cultures de cet organe ont révélé le *Corynebacterium pseudotuberculosis murium* (KUTSCHER), qui, pour différents motifs, a été considéré comme l'agent de la maladie. Ce micro-organisme n'a provoqué les pneumonies à foyers, régulièrement décrites par la bibliographie, que lorsqu'il a été administré oralement avec les aliments.

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(From the Laboratory of the Waterworks, Rotterdam).

# AN IMPROVEMENT IN THE BACTO-TRYPTONE, SODIUM FORMATE MEDIUM FOR THE DETECTION OF *B. COLI*

by

**T. FOLPMERS**

(Received December 20, 1943).

An improvement of the bacto-tryptone, sodium formate medium [1]), which has now been put on trial for nearly a year in the Laboratory of the Waterworks at Rotterdam, consists in lowering the content of crystal violet to half of the original amount and in raising the pH to 6.8.

The medium is now prepared as follows : 0.3 % bacto-tryptone, 0.04 % $K_2HPO_4$, 0.1 % HCOONa, 0.00005 % crystal violet, pH = 6.8. Dissolve in 1800 ml distilled water 30 grams bacto-tryptone, 4 grams $K_2HPO_4$ and 1.3 grams NaCl. Filter through Swedish filterpaper, add ± 13 ml n HCl. Sterilise in Koch's steriliser for an hour. Add 50 ml sterilised 20 % sodium formate solution, 5 ml sterilised aqueous crystal violet solution (1 : 1000) and sterile distilled water up to 2000 ml.

Cultivate in completely filled stoppered glass bottles at 44°—45° C. in a 1 : 5 dilution of the medium. For this purpose supply the glass bottle with one fifth of its content with the culture medium, add the water to be tested either undiluted or in known dilution and fill in the latter case the bottle with sterile water. Test for indol and gas production after 24 and 48 hours. Gas production is often very slight; in such case attention has to be paid to an eventual growth of coli bacteria at the bottom of the glass bottle, which may be rendered visible by shaking the bottle gently.

Indol reagents : 2 grams p. dimethylamino-benzaldehyde in 190 ml 90 % alcohol and 40 ml 25 % HCl. Bring the reagent by means of a pipette as a thin layer over an aliquot of the culture solution in a test tube and heat this in the small flame of a Bunsen burner.

1) cf. T. FOLPMERS, Antonie van Leeuwenhoek **6**, 22, 1939—1940.

(From the Department for Bacteriology and Experimental Pathology of the Institute of Preventive Medicine at Leiden).

# A FILTERABLE VIRUS AS A CAUSATIVE AGENT OF EPIDEMIC HEPATITIS

by

**J. D. VERLINDE** and **A. J. VAN DEN HOVEN VAN GENDEREN**

(Received January 14, 1944).

## 1. Introduction.

Many cases of so-called catarrhal jaundice are based on diffuse hepatitis. This disease, which can spread epidemically and especially in war time pandemically, is indicated by the name of epidemic hepatitis. Though its cause is not known, it is surmised that the agent is a filterable virus. Several arguments support this conception. The sometimes epidemical spreading, especially in winter, points to the infective nature of the affection. Nobody succeeded however, in pointing out visible micro-organisms as a cause, nor did the serological investigations with respect to the known pathogenic ones, among which we especially think of leptospirae, give any indications as to the presumable etiology. Attempts to transmit the disease on animals by inoculation with blood, urine or duodenal fluid failed with hardly any exception. Of late years however, some facts have been observed that might point to a virus etiology. For epidemic hepatitis has indeed been diagnosed after the injection of sterile human serum (Findlay, Mc. Callum and Murgatroyd (5)), and after blood transfusion (Junet, l.c. Ziegler (10)). After the inoculation of duodenal fluid of sufferers from epidemic hepatitis on the chorioallantois of the chick embryo, Siede and Meding (7) and Siede and Luz (6) made evident the existence of a filterable agent, which they could grow on in eight egg passages and which regularly caused the death of the embryos after five days.

Voegt (9) tried to infect some persons by making them drink the duodenal fluid of sufferers from epidemic hepatitis or by subcutaneous injections of blood or urine. He did not succeed however, in exciting the typical clinical picture of the disease; particularly jaundice was always absent. He communicated, that after about four weeks a slight damage of the liver occurred, which was stated by burdening experiments and by chemical analyses of blood and urine.

Interesting but not yet proved experiments have been made by Andersen and Tulinius (1,2 ,3). They start from the supposition

that the enzootic hepatitis of pigs, which is of frequent occurrence in Denmark, has perhaps a causal connection with epidemic hepatitis of man. They base this hypothesis on the conformity of the histopathological changes in the livers in both diseases and on the fact that the epidemics of hepatitis in man occur along with those in pigs, which parellelism they prove by means of diagrams. At Copenhagen e.g. where the meat inspection is very severe, all icteric pigs are declared unfit for human consumption. In the country and in most other towns this is not the case and it is true that there epidemic hepatitis is much more frequent than at Copenhagen. In the case of one epidemic, they suggest to have indicated a butcher's shop, where the meat of an icteric pig had been sold, as a source of infection.

They tried to prove their hypothesis experimentally and suggest to have succeeded in transmitting the hepatitis of pigs on young and underfed pigs by feeding them with liver. Jaundice would appear already three days after the inoculation. Infection would also be possible by drinking duodenal fluid of diseased pigs. By oral inoculation they could transmit the hepatitis of pigs in four and the hepatitis of man in two passages. In both cases the histological changes of the liver agreed with those of the spontaneous cases in man and pigs. The bacteriological investigations always having a negative result, they suggest to have proved, that the epidemic hepatitis of man and pigs is caused by the same virus.

In this war, epidemic hepatitis appears again pandemically and in the winter of 1942/1943 we set up an investigation into the etiology of the disease. The result of this was, that by means of experiments on guinea pigs a filterable virus could be shown as a cause, which has already been briefly communicated elsewhere (Verlinde and Boer.(8)). Without going further into the clinical picture of the patients, we shall dwell a little longer on the experimental investigations, which have again been extended since the preceding publication.

2. Inoculation experiments in guinea pigs.

So far inoculation experiments have been made with the material of eight patients suffering from epidemic hepatitis. In four of them we succeeded in demonstrating the virus in the blood during the initial fever as well as in the urine during the jaundice (see table 1).

As a rule we used the intraperitoneal, in guinea pig 160 and 162 the intracardial inoculation. The temperature was taken twice a day, *viz.*, at 9 o'clock a.m. and at 4 o'clock p.m. The guinea pigs that reacted positively upon the injection, showed a rise of temperature which was not very conspicuous, except the animals injected with the urine of patient 5. Sometimes only the afternoon temperature gave a distinct top. Once or twice a second top was observed some days afterwards (fig. 1).

Table 1.

| Patient number | Material | Stage of the disease | Inoculated guinea pig | Reaction | Incubation period |
|---|---|---|---|---|---|
| 5 | urine (unfiltered) | jaundice 4 days | 159 | + | 8 days |
| | ,, | ,, | 160 | + | 8 ,, |
| | urine (Seitz filtrate) | ,, | 161 | + | 8 ,, |
| | ,, | ,, | 162 | + | 8 ,, |
| 6 | blood (defibrinated) | fever | 190 | ± | 10 days |
| | ,, | ,, | 191 | — | |
| 7 | urine (unfiltered) | jaundice 1 day | 217 | — | |
| | ,, | ,, | 218 | — | |
| | urine (filtered) | ,, | 219 | — | |
| | ,, | ,, | 220 | — | |
| 10 | blood (defibrinated) | fever | 273 | + | 7 days |
| | ,, | ,, | 274 | + | 7 ,, |
| | urine (unfiltered) | jaundice 2 days | 309 | — | |
| | ,, | ,, | 310 | + | 6 ,, |
| 12 | stools (Seitz filtrate) | jaundice | 353 | — | |
| | ,, | ,, | 354 | — | |
| 14 | liver punctation (post | liver atrophy | 287 | — | |
| | ,, (mortem) | ,, | 288 | — | |
| 15 | urine (unfiltered) | jaundice | 361 | — | |
| | ,, | ,, | 362 | — | |
| 16 | urine (unfiltered) | jaundice 2 days | 423 | — | |
| | ,, | ,, | 424 | + | 10 days |
| | throat-swill (Seitz | ,, | 425 | — | |
| | ,, filtrate) | ,, | 426 | — | |

On judging the temperature the course of the normal curve must be taken in consideration for each animal individually, so that it is advisable to registrate the temperature already some time before the injection. The normal temperature is not the same in every

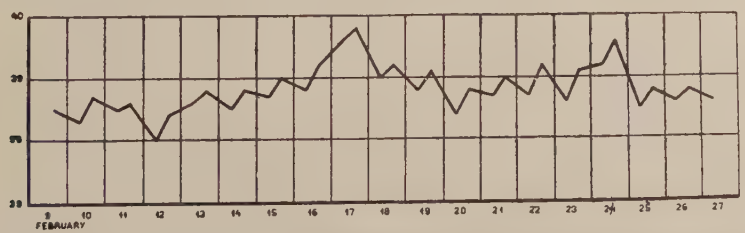


Fig. 1. February 9. intraperitoneal inoculation urine patient 5 (Seitz filtrate).

guinea pig and also in one single animal it may vary. On the day after the injection a certain rise of temperature is sometimes perceptible, which must be understood however, as a non-specific reaction to the injection. Generally this rise does not last longer than one day. For each inoculation at least two guinea pigs have

been used. One of them was killed when the temperature had risen considerably, in the other we watched the further course, and afterwards the animal could be used for immunity experiments. In the killed guinea pig, the organs were bacteriologically investigated, after which they could be used for inoculation experiments, when the result had been negative.

In order to be able to exclude in the blood and in the urine of normal persons a possible presence of an agent that might cause the same reaction in guinea pigs, we gave eight guinea pigs an intraperitoneal injection with 5 ml of the blood and the urine of four normal persons. This did not cause rises of temperature and inoculation experiments with the blood and the organs of three of them, which had been killed after 6, 8 and 10 days, had a negative course.

When the result of the bacteriological investigations of the killed guinea pigs was negative, the inoculation experiments were carried out on fresh guinea pigs. In this way, starting from patients numbers 5, 6, 10 and 16, respectively 11, 8, 6 and 7 passages were made. In the passage animals fever as a rule is evident; the temperature curve often shows two tops, sometimes even a period of fever, which may last mostly 3—5 and once 12 days (fig. 2 and 3).

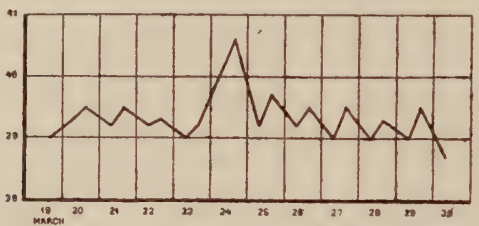

Fig. 2. Guinea pig 232: March 19. intraperitoneal inoculation blood plasma of guinea pig 209.

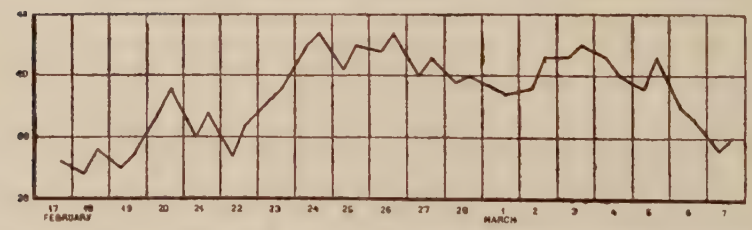

Fig. 3. Guinea pig 166: February 17. intraperitoneal inoculation liver guinea pig 159.

It may occur, as was the case in patients 6 and 10, that the guinea pigs, after the injection of urine or blood of a patient, do not react distinctly by a rise of temperature, or do not react at all. When the animals however, are killed 8—10 days after the injection, fever does occur in the second passage. Therefore it is possible that not all guinea pigs giving a negative reaction, are actually negative, but

that they might become positive as yet by one or more blind passages. Also in series inoculations it occurs repeatedly that only one of the two inoculated animals reacts with fever, whilst the next passage of such a clinically negative animal may be distinctly positive. So in fact the guinea pig is sensitive to the virus of epidemic hepatitis, but not in a high degree. We found young guinea pigs of a weight of 250—350 g the best suited for experimental researches with the virus.

The inoculation experiments have been made with blood, organs and bile during the fever period and with urine after this period. The blood has been taken by puncture of the heart; the organ suspensions have been prepared by grinding the organ with sterile sand and suspending it in 9 parts of a 0,9 % saline solution. The Seitz EK filtrate has been made of organ suspensions prepared in the way described above. The quantity of blood or of organ suspensions (unfiltered) injected, amounts to 1—5 ml, whilst we generally injected 5 ml of the Seitz filtrate. It appears from table 2, that the virus could be ascertained in the blood and in the organs during the fever period. After this the virus is apparently excreted with the urine during merely a short period, for in the guinea pig it could still be shown in the urine 5 days after the fever period, a fortnight after this period no more.

Table 2.

| Material | number of guinea pigs | |
|---|---|---|
| | infected | positive |
| fresh blood | 5 | 3 |
| oxalated blood | 5 | 3 |
| red blood corpuscules | 2 | 1 |
| bloodplasm | 2 | 2 |
| liver (unfiltered) | 50 | 32 |
| liver (Seitz filtrate) | 3 | 2 |
| liver (dried) | 2 | 2 |
| kidney | 2 | 1 |
| spleen | 2 | 2 |
| brain | 2 | 1 |
| lung | 1 | 1 |
| bile | 2 | 0 |
| urine (5 days after fever) | 1 | 1 |
| urine (14 days after fever) | 3 | 0 |

In patient 10 we did not succeed in demonstrating the virus in the urine a week after the cure of jaundice. In patient 16, on the other hand, the virus was still present in the urine a week after this cure.

The method of inoculation generally followed, is the intraperitoneal one, but it is also possible, though less constant in result, to infect guinea pigs by the intracardial, subcutaneous, oral and intracerebral route. The average incubation period after the in-

traperitoneal, intracardial and subcutaneous inoculation amounts to 6—8 days. The shortest interval observed is 3 days. After the oral inoculation (the animals being fed with virulent liver of guinea pigs), it amounted to 13 days. Twice we tried to effect an infection by contact by putting a guinea pig in a hutch together with a guinea pig with fever. One of these animals reacted with fever after 15 days. The length of this period agrees with the incubation period given for man (2—4 weeks).

3. Post mortem examination in the guinea pig.

The disease in the guinea pig seldom has a fatal course. In the few guinea pigs that died and in those, killed at different moments after the inoculation, we mostly did not find macroscopical changes. During the fever period the spleen may be swollen. Then the liver too is sometimes swollen and its surface may have a light yellow spotted aspect. In some cases after the fever period gray or white-yellow to ochreous, irregular foci are found, which on histological examination correspond with foci of atrophy and necrobiosis. Once we found a diffuse atrophy of a whole liver lobe, which had a yellow aspect. The focal changes are intralobular, may expand over the whole lobulus and sometimes fuse with the neighbouring lobuli, showing the same alterations. The process begins with swelling and dissociation of liver cells, and vacuolisation of the protoplasm. The taking of the stain by the protoplasm grows less and may disappear nearly completely, so that the focus assumes a transparent aspect.

Subsequently a diminuation of the liver cells may occur. The nuclei mostly remain unimpaired, but sometimes symptcms of degeneration occur. In or around the foci, sometimes through the whole liver, a fatty infiltration can be found. The cellular reaction is not conspicuous and consists of a slight perilobular infiltration with lymphocytes, which sometimes may be found on the edge of the foci. In the foci, the number of Kupffer cells may be increased. In the spleen, many megacaryocytes and plasmacells may be present, the reticulum often being loaded with little drops of fat. In the kidneys slight degenerative changes of the epithelium of the tubes are found.

We want to point to the fact that some of these changes may be absent. It may even occur that the pathological findings are perfectly negative. On the other hand we sometimes saw the changes in the liver described in guinea pigs that had not reacted to the inoculation with fever. The changes found by us in the liver of the guinea pig agree with those described in man.

4. Inoculation experiments in mice and rats.

When a guinea pig virus is transmitted to mice and rats either by the intraperitoneal, subcutaneous, intracerebral or oral route, no clinically perceptible morbid symptoms of fever appear, nor do we see typical alterations on autopsy. The virus however, may remain active when inoculated in series once a week. Usually the liver is

also used for the inoculations. As in these animals no criteria are observed that would prove the presence of the virus, it is only possible to demonstrate the virus by the inoculation of guinea pigs. Attempts to obtain a neurotropic virus by intracerebral route, such as is proceeded for the neurotropic virus of yellow fever, have not had any result so far. It has been possible to grow the virus on mice and rats during 5 passages, in the passage following this could not be demonstrated any more. Starting however from the virus of egg membrane eight cerebral mouse passages could be made so far.

5. Properties of the virus.

In 50 % glycerol the virulence can be retained at a temperature of 0—4° C. for 1—3 months. One strain was still virulent after 83 days, another having lost its virulence already after 51 days.

Dried virus, which is obtained by grinding the fresh liver of a guinea pig killed during the fever period and drying it in a vacuum exsiccator with sulphuric acid and anhydrous phosphoric acid at 4° C., proved to be still virulent after having been kept in a refrigerator for 33 days; after 63 days it had lost its virulence. When however, the virus is kept at —16° C., the virulence can be retained for at least half a year.

With a virulent suspension of liver, which has been heated at 55° C. for half an hour, it is not possible to excite a reaction in guinea pigs. So the virus soon looses its virulence by heat.

After the action of 0.1 % formaline at 37° C. a liver suspension was still able to excite fever in the guinea pig. This was not possible in a growth on egg membrane. After the liver suspension had been kept at 4° C. for another week, the virus proved to be inactivated.

The virus can easily be filtrated through a Seitz EK filter. Already in the first experiments we succeeded in exciting fever with filtrated urine, whilst afterwards it has also appeared that filtrated suspensions of liver and of egg membrane could excite this reaction as well. It is true, that the filtration is attended with a loss of virulence, which a longer incubation period makes apparent as well as with a shorter fever period after the inoculation with filtrated material in comparison with the same, but unfiltrated material.

Generally the virulence to the guinea pig is not high; 57 % out of about 100 passage guinea pigs reacted with distinct fever, 18 % reacted doubtfully and 25 % did not react at all.

We believe to have traced in our experiments a certain influence of the season on the sensitiveness of the guinea pig. In the winter months the inoculations give a more conspicuous reaction, while in summer they become effective with the greatest difficulty and only a strain of strongly virulent material can maintain itself in summer, whilst the less virulent ones are lost. These observations agree with the epidemiology in man; epidemic hepatitis occurs especially in winter.

6. The growth of the virus on the egg membrane.

We have tried to grow the virus on the chorioallantois of the chick embryo, according to the method of Burnet (4). After an incubation time of 11 days, the eggs are inoculated, for which we used an artificial air space; the final inoculation taking place after three more days. We started from a virulent liver of a guinea pig out of the series of patient number 5. About 20 egg passages have been made and the virus can still be demonstrated in the egg membrane and, though less apparent, in the liver and the brain of the embryo, by inoculation of the guinea pig (table 3).

Table 3.

| Egg passage | | Guinea pig | Reaction | Incubation period |
|---|---|---|---|---|
| Passage-number | Material | | | |
| 4 | embryo liver | 292 | + | 5 days |
| | „ | 304 | + | 7 „ |
| | allantois | 313 | — | |
| | „ | 335 | + | 8 „ |
| | „ | 336 | — | |
| 7 | embryo liver | 347 | + | 4 „ |
| | „ | 348 | — | |
| | allantois | 349 | + | 12 „ |
| | „ | 350 | + | 8 „ |
| 11 | „ | 371 | — | |
| | „ | 372 | — | |
| | „ | 373 | + | 4 „ |
| 14 | embryo liver | 405 | + | 10 „ |
| | „ | 406 | — | |
| | embryo brain | 407 | — | |
| | „ | 408 | + | 7 „ |
| 16 | allantois | 415 | + | 5 „ |
| | „ | 416 | + | 8 „ |
| | „ | 417 | + | 5 „ |
| | „ | 418 | + | 5 „ |
| 20 | „ | 537 | + | 5 „ |
| | „ | 538 | + | 7 „ |

Also the direct inoculation of eggs with filtrated urine (patient 16) has now been succesful.

Contrary to the results of Siede c.s. (6, 7) our infected embryos as a rule do not die, at least not within 5 days after the inoculation.

Apparent changes, besides hyperaemia, sometimes local edema and small hemorrhages have not been found so far on the egg membranes. In the smears of the allantois, which have been stained by the Gram or Giemsa-method, we sometimes found in and around the cells large quantities of small round strongly coloured bodies, which have not been found in normal preparations. It is not improbable that these are elementary bodies of the virus.

A virulence experiment has been carried out with the 16th allantois-passage. The allantois was ground and diluted with a 0,9% saline-solution till 1/10, 1/100, 1/1000, 1/10.000 and 1/100.000. Of each dilution 5 ml has been intraperitoneally injected into two guinea pigs. The dilution of 1/1000 still caused some rise of temperature. This was not the case with higher dilutions.

7. Immunity.

Guinea pigs that are infected with virulent material for the second time, do not react unless they are entirely recovered from the first infection. The moment in which the state of immunity sets in seems not only to depend on the date of the first infection, but also on the length of the fever period (Table 4).

Table 4.

| Guinea pig. | Time between the first infection and the reinfection | Time between the last day of fever and the reinfection | Immunity |
|---|---|---|---|
| 161 | 20 days | 5 days | — |
| 162 | 20 „ | 12 „ | + |
| 171 | 34 „ | 25 „ | + |
| 178 | 26 „ | no fever | + |
| 187 | 22 „ | 15 days | + |
| 191 | 21 „ | 14 „ | + |
| 198 | 19 „ | 12 „ | — |
| 216 | 31 „ | 26 „ | + |
| 222 | 25 „ | no fever | + |

All guinea pigs mentioned in Table 4 have been reinfected with the same infectious material (liver of guinea pigs), which in normal guinea pigs excited a clear-cut fever reaction.

The first infections of guinea pigs 161 and 162 took place simultaneously. Both are reinfected 20 days later, but only guinea pig 162 is immune. The difference between the two animals consists in number 161 having had a long fever period and number 162 a short one, so that the reinfection of number 161 took place already 5 days after the last day of fever, whilst in number 162, 12 days passed between the last day of fever and the reinfection. Guinea pig 198, in the case of which also 12 days passed between the last day of fever and the reinfection, is not yet immune. So the shortest lapse of time after which a distinct immunity can be shown, must be rated at 12 days after the period of fever. The other guinea pigs have not reacted to the reinfection and so they are probably immune.

We cannot be certain whether this is really the case for we have noted that in normal guinea pigs the infection becomes effective only for 57 % as well. The fact, however, that a number of these animals did react with fever to the first inoculation, prove that they have been sensitive to the virus. Their not being sensitive to the

second infection points to an immunity obtained as a result of the first infection.

When the first infection has not excited fever, as in numbers 178 and 222, even then immunity may appear. This can also be explained by the animals being already naturally immune to the virus. So it is difficult to make out here whether the immunity is a result of the first infection. Yet this is probable, at least in one guinea pig, as neutralizing antibodies have been shown in the blood serum and these do not occur in the normal guinea pig.

For the detecting of neutralizing antibodies three or more suspensions of a virulent liver of a guinea pig are made, e.g. in the dilution 1/10, 1/20 and 1/50. To 1 ml of the virus dilution 1 ml of undiluted serum that is to be investigated, is added. The mixtures are kept at 37° C. for 2 hours and at 4° C. for half an hour; after that they are intraperitoneally injected into guinea pigs. In order to control the activity of the virus a same number of virus dilutions is mixed with a dilution of 0,9 % saline solution, which mixtures are treated in the same way. The temperature of the guinea pigs was taken twice a day during a fortnight. When the guinea pigs which have been injected with the virus serum mixtures react with fever, we can conclude that there are no antibodies. If the controls do react and these animals not, it is very probable that the serum contains antibodies.

However, as long as we do not succeed in obtaining a virus-fixe which excites fever in every case, the neutralization experiment cannot be considered as sufficiently reliable. We had hoped to find the mouse an animal suited to the neutralization experiment, but in this animal no typical reaction occurs from which the result of the experiment could be concluded. The results of the neutralization experiments with sera of patients and guinea pigs has been summarized in table 5. The virus dilutions used were 1/10, 1/20 and 1/50. With each mixture two guinea pigs were injected, so for every serum 12 guinea pigs have been used (6 for the virus-serum mixtures, 6 for the control mixtures).

It appears from table 5 that the neutralizing antibodies can be traced from the 13th day of the fever-period, *i.e.*, nearly simultaneously with the appearance of the resistence to the reinfection.

Guinea pigs that have not reacted with fever may yet possess antibodies. Some sera of patients showed a weak complement-fixation-reaction with an antigen prepared by keeping a 10 % suspension of a fresh virulent liver of a guinea pig at 37° C. for 24 hours and at 4° C. for two more days, and subsequently centrifugating it at 3000 r.p.m. for 15 minutes.

The technics used for the reaction were those of BESREDKA. The results are expressed in the number of unities of complement. One unity of complement is 0.1 ml of fresh guinea pig serum 1/15 which is fixed by 1 ml of the patient-serum. Patients-sera 1, 6, 7, 8, and 14 fixed respectively 10, 15, 15, 20 and 10 unities, 5 normal sera fixed 0 unities.

Table 5.

| Serum | | Number of days after the period of lever | Result of the experiment |
|---|---|---|---|
| Patient | 1 | 90 | +++ |
| „ | 5 | 24 | ++ |
| „ | 6 | 18 | ++ |
| „ | 7 | 17 | + |
| „ | 8 | 36 | ++ |
| „ | 9 | 25 | +++ |
| „ | 14 | unknown | ++ |
| Guinea pig | 160 | 5 | — |
| „ | 166 | 36 | ++ |
| „ | 177 | no reaction | + |
| „ | 179 | 16 | + |
| „ | 193 | 13 | + |
| „ | 208 | 26 | +++ |
| „ | 215 | no reaction | — |
| „ | 221 | „ | ++ |
| „ | 222 | „ | + |
| „ | 223 | „ | + |
| „ | 226 | 21 | ++ |
| „ | 227 | 15 | ++ |
| „ | 229 | 22 | + |
| „ | 233 | 14 | ++ |
| „ | 243 | 14 | + |

— all mixtures positive (no neutralization).
+ mixtures 1/10 and 1/20 positive (weak neutralization).
++ mixtures 1/10 positive (moderate neutralization).
+++ all mixtures negative (strong neutralization).

## Summary.

In four patients suffering from epidemic hepatitis we succeeded in isolating from the blood during the fever period and from the urine during the jaundice, a filterable virus, which is pathogenic to the guinea pig and which can be inoculated to this animal in different ways, but by preference intraperitoneally. Fever, which sometimes lasts only one day, is the only morbid symptom observed in guinea pigs.

In these animals the virus can be shown in the organs and in the blood during the fever period; after that it is excreted with the urine during apparently a short period. The virus has been grown on the chorioallantois of the chick embryo so far during 20 passages. The virus is resistant to glycerol, drying and low temperatures, not to formaline and heating.

In the livers of the guinea pigs focal changes (degeneration, dissociation, necrobiosis, yellow liver-atrophy) may be found. After pulling through the disease immunity occurs, whilst in the serum of recovered patients and guinea pigs neutralizing antibodies can be detected.

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(Aus dem Centraalbureau voor Schimmelcultures, Baarn).

# BESCHREIBUNG EINIGER NEUER PILZARTEN AUS DEM CENTRAALBUREAU VOOR SCHIMMELCULTURES, BAARN (NEDERLAND)

## VIII. MITTEILUNG

von

**F. H. VAN BEYMA THOE KINGMA**

(Eingegangen am 7 Februar, 1944).

### *Chaetomium minimum* nov. spec.

Dieser Pilz wurde dem C.B.S. von Herrn Ir. E. HARMSEN (Kampen) zugeschickt, der denselben aus sauren Podsol-Böden wiederholt isolierte. Vor allen Dingen BAINIER (2) und später CHIVERS (6) haben sich mit dem Studium der Gattung *Chaetomium* befasst und zahlreiche Arten unterschieden und beschrieben. Unser Pilz konnte jedoch mit keiner der dort aufgezählten oder in der Sammlung des C.B.S. vorhandenen Arten identifiziert werden. Wegen der geringen Grösse der Perithezien haben wir ihn *Ch. minimum* genannt.

Die Perithezien sind ziemlich klein, kleiner wie es bei den meisten Arten dieser Gattung gewöhnlich der Fall ist. Die grösste Aehnlichkeit zeigt unser Pilz noch mit *Ch. aureum* Chivers; von diesem unterscheidet er sich jedoch durch die schwarze Farbe der reifen Perithezien und die grösseren, ausserdem abweichend gestalteten Sporen. Die Grösse der Askosporen beträgt bei *Ch. minimum* im Mittel 11,9 × 5,6 $\mu$, bei *Ch. aureum* 9,8 × 5,4 $\mu$. Bei letztgenannter Art sind sie mehr oder weniger oval, bei erstgenannter spindelförmig, oft mit einer geraden Seite. Sie treten aus den Perithezien in geraden oder gekrümmten, bis


Fig. 1. *Chaetomium minimum*.
*a*. Perithezien. Vergr. 80 ×.
*b*. Asci. Vergr. 750 ×.
*c*. Askosporen. Vergr. 750 ×.

40 $\mu$ breiten Säulen hervor. Der Haarschopf der Perithezien fällt nicht besonders auf, er besteht meist aus einigen steifen, braunen, inkrustierten, 4 $\mu$ dicken Borsten, welche an der Spitze bischoffsstabähnlich umgebogen sind.

Auf Agar-Nährböden bildet der Pilz wollige bis strähnig-wollige, gelbbraune Decken, wobei ein in den Nährboden hinein diffundierender Farbstoff denselben dunkelbraun bis fast schwarzbraun färbt. Das beste Wachstum erzielt man auf Lupinenstengeln, diese sind innerhalb einer Woche von zahlreichen Perithezien überwachsen, ohne Luftmyzel.

In bestimmten Böden scheint *Ch. minimum* eine wichtige Rolle zu spielen. In einem Schreiben an das C.B.S. teilt Ir. E. Harmsen mit, dass dieses *Chaetomium* der wichtigste Zellulose-Zersetzer in sauren Podsol-Böden während der ersten Phase des Abbruchs von frisch untergepflügtem, zellulosereichem Material (Stroh, Spreu, Papier, Abfälle, u.s.w.) in den Niederlanden sei; sie enthalten bisweilen bis zu 20.000 Keime/Gramm.

Die Beschreibung lautet wie folgt:

*Chaetomium minimum* nov. spec.

*Perithezien* braunschwarz bis schwarz, (120—130) × (70—100) $\mu$, ellipsoidisch bis gedrückt-kegelförmig. Haarschopf aus verhältnismässig spärlichen, septierten, dunkelbraunen, steifen, inkrustierten, 2—3 $\mu$ dicken Borsten bestehend, welche an der Spitze bischoffsstabähnlich umgebogen sind. Rhizoiden spärlich entwickelt, seitliche Haare einfach, pfriemenförmig, septiert, braun, inkrustiert, 4 $\mu$ dick.

*Asci* keulenförmig, kurz gestielt, vergänglich, 36—45 $\mu$ lang (pars sporif. 33 $\mu$) und 10—12 $\mu$ breit.

*Sporen* spindelförmig, oft mit einer geraden Seite, olivenbraun, (10,7—13,3) × (5—6) $\mu$ — meist (12—13) × (5—5,7) $\mu$ — in langen, schwarzen, bis 40 $\mu$ dicken, geraden oder gekrümmten Säulen austretend.

*Hab.* Aus sauren Podsol-Böden aus der Gegend von Kampen (Niederl.) (Ir. E. Harmsen).

Lateinische Beschreibung.

Peritheziis atrobrunneis vel atris, minutis, (120—130) × (70—100) $\mu$ ellipsoideis vel obtusiter coniformibus. Setis raris, rigentibus et atrobrunneis in acumine curvis. Pilis rhizoideis modicis. Filis lateralibus subuliformibus brunneis. Ascis fustiformibus, in brevibus pediculis sedentibus, fragilibus 36—45 $\mu$ longis (pars sporif. 33 $\mu$), 10—12 $\mu$ latis. Sporulis fusiformibus saepe inaequilateralibus, colore brunneo oleagino, (10,7—3,3) × (5—6) $\mu$, — plerumque (12—13) × (5—5,7) $\mu$ —, se exserentibus stylis longis, usque ad 40 $\mu$ latis, distichis.

***Piptocephalis macrospora* nov. spec.**

Aus dem Laboratorium der „Specerijen-Malerij De Kō ver" in Boxmeer (Niederl.) erhielt das C. B. S. verschimmelten Nelkenersatz

zur Untersuchung. Aus dem mit Wasser zu einer feuchten Masse verrührten Pulver, das auf feuchtem Filtrierpapier in einer Petrischale einige Tage bei 24° C. gehalten wurde, entwickelte sich ein Pilz, der alsbald als zur Gattung *Piptocephalis* gehörig erkannt werden konnte. Da *Piptocephalis* bekanntlich auf anderen Mucorineen schmarotzt, konnte die Anwesenheit von Pilzen dieser Familie vermutet werden. Bei der Abimpfung wurden in der Tat zwei Mucorineen in den Kulturgefässen festgestellt und zwar *Mucor racemosus* Fres. und *Absidia ramosa* (Lindt) Lendner. Als einer der am meisten geeigneten Nährböden erwies sich sterilisierte Möhre. Die Herstellung von Reinkulturen gelang leicht, da die Sporangienträger von *Piptocephalis* sich ziemlich hoch über die Pulvermasse erheben, sodass mit Hilfe einer Pinzette die Spitzen der Träger leicht erfasst und auf Möhre übertragen werden konnten. Die Fruchthyphen des Pilzes bilden weder Rhizoiden noch Ausläufer; sie entspringen unmittelbar aus dem Myzel. Die Verzweigung der Sporangienträger ist die für *Piptocephalis* typische, nämlich wiederholt dichotom. Die septierten Gabeläste sind anfangs hyalin, werden aber bald braun und weisen eine feine Längsstreifung auf. Die letzten Verzweigungen enden in Zellen, die wir nach ZYCHA Basalzellen nennen wollen. Diese Basalzellen sind kugelig, 7—8 $\mu$ im Durchmesser und ringsum mit zahlreichen Höckerchen, woran die Teilsporangien sitzen, versehen. Sie fallen leicht ab, sodass man sie überall im mikroskopischen Präparat umherschwimmen sieht.

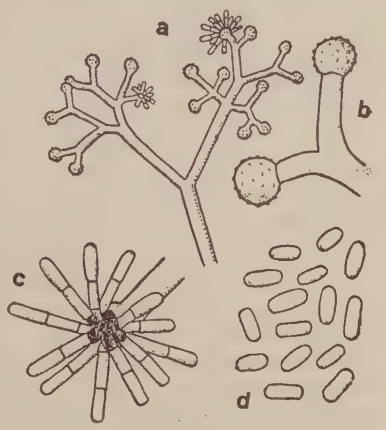


Fig. 2. *Piptocephalis macrospora.*
*a.* Sporangienträger. Vergr. 245 ×.
*b.* Basalzellen. Vergr. 750 ×.
*c.* Köpfchen. Vergr. 750 ×.
*d.* Sporen. Vergr. 750 ×.

Was die systematische Stellung anbetrifft steht unser Pilz zweifellos der *Piptocephalis cylindrospora* nahe. Der Unterschied besteht hauptsächlich hierin, dass bei unserem Pilze die Grösse der Basalzellen 7—8 $\mu$, die der Sporen 7 × (3—3,3) $\mu$ beträgt, gegen 3—4 $\mu$ für die Basalzellen und 4 × 2 $\mu$ für die Sporen bei *Piptocephalis cylindrospora*. Auf Grund dieser grösseren Abmessungen haben wir den Pilz *Piptocephalis macrospora* genannt (9, 11).

Die Beschreibung des Pilzes gestaltet sich folgendermaassen:

*Piptocephalis macrospora* nov. spec.

*Myzel* ohne Ausläufer.

*Sporangienträger* aufrecht, mehrfach dichotom verzweigt, mit rechtwinklig abzweigenden, nach oben immer kürzer werdenden Gabel-

ästen, anfangs farblos, später braungelb mit undeutlich gestreifter Membran, septiert, mit farblosem Inhalt.

*Basalzellen* kugelig, 7—8 $\mu$ im Durchmesser, allseitig mit kleinen Höckerchen versehen, leicht, jedoch nicht immer, abfallend.

*Teilsporangien* zahlreich, etwa 15—20 $\mu$ lang, 2—3 gliedrig, gerade, aufrecht.

*Sporen* zylindrisch; hyalin, glatt, (6,3—8,7) × (3—3,3) $\mu$, meist 7 × (3—3,3) $\mu$.

*Zygosporen* unbekannt.

*Hab.* Parasitisch auf *Mucor racemosus* Fres. und *Absidia ramosa* (Lindt) Lendner, wachsend auf Nelkenersatz (Specerijen-Malerij De Körver, Boxmeer, Niederl.).

Lateinische Beschreibung.

Mycelio sine ramis. Conidiophoris erectis, saepe dichotomis, primo sine colore, deinde brunneoflavis, membrano obscuriter striato praeditis. Cellulis basalibus globosis, 7—8 $\mu$ crassis. Sporulis cylindriformibus, hyalinis, levibus, (6,3—8,7) × (3—3,3) $\mu$; plerumque 7 × (3—3,3) $\mu$. Zygosporula ignota sunt.

## *Scopularia corsicana* nov. spec.

Von Frl. Dr. H. C. Koning (Baarn) erhielt das C. B. S. einen Pilz, isoliert aus dem Holze von *Pinus nigra corsicana*. Es handelte sich um etwa dreijährige Kiefer mit wahrscheinlich durch Frost beschädigten Trieben, deren Nadeln an den Spitzen braun verfärbt waren. Im Verlauf desselben Jahres hatten die Bäumchen sich jedoch völlig erholt.

Der betreffende Pilz wurde leicht als zur Gattung *Scopularia* Preuss em. G. Goidànich (Syn. *Leptographium* Lagerberg et Melin) gehörig erkannt. Die Merkmale: Konidienträger aus einem einzelnen Faden aufgebaut mit dunkelbraunem Stiele, der in einen penicilliumartig verzweigten Pinsel übergeht und die zu grossen Tropfen zusammenfliessenden Konidien waren sämtlich anwesend. G. Goidànich (8) hat die Gattung *Grosmannia* gegründet als perfekte Form von *Scopularia*. Eine Askus-Form ist jedoch bis jetzt in unseren Kulturen nicht aufgetreten. Der Pilz konnte mit keiner der beschriebenen oder in der Sammlung des C.B.S.


Fig. 3. *Scopularia corsicana*.
*a*. Konidienträger. Vergr. 245 ×.
*b*. Endästchen der Konidienträger. 750 ×.
*c*. Konidien. Vergr. 750 ×.

vorhandenen *Scopularia*-Arten identifiziert werden. Von den ihm am nächsten stehenden Arten: *Sc. tenuissima* (Cda) G. Goid., *Sc. penicillioides* (Grosm.) G. Goid., *Sc. Lundbergii* (Lag. et Melin) G. Goid., oder *Sc. pini* G. Goid. weicht er entschieden ab. *Sc. tenuissima* besitzt spindelförmige Konidien, *Sc. penicillioides* gekrümmte Konidien, *Sc. Lundbergii* weist grössere Konidien auf und *Sc. pini* endlich hat anders gestaltete Konidienträger.

Er wurde deshalb als neue Art beschrieben. Die Beschreibung lautet:

*Scopularia corsicana* nov. spec.

*Hyphen* von kräftigem Wuchs, 2,7—3 $\mu$ breit, septiert, leichtbraun gefärbt, im Alter nachdunkelnd bis fast schwarz.

*Konidienträger* 4—6 $\mu$ breit, mit braun gefärbtem Stiel, aufrecht, septiert, nur am Scheitel mehrfach verästelt. Endästchen 10—16 $\mu$ lang und 2 $\mu$ breit, hyalin bis leicht gefärbt, zahlreich, dicht gedrängt stehend und die Konidien in grossen Massen abschnürend.

*Konidien* massenhaft, in feuchter Umgebung zu grossen schleimigen Massen zusammenfliessend, einzellig, ellipsoidisch, von verschiedener Grösse, (4—12) × (2—4,7) $\mu$, meist (5,3—6,3) × (2,7—3,3) $\mu$, ohne Oeltröpfchen, jedoch mit einer Vakuole oder mit undeutlichem Inhalt.

*Hab.* Aus dem Holze von *Pinus nigra corsicana* (Frl. Dr. H. Koning, Baarn).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 6 Tagen: schnellwüchsig, Schale ganz bewachsen, vom Impfstück aus flach dem Agar anliegende schwarze Hyphenstränge. Ueberwuchs von kleinen, wolligen, hellbräunlichen Myzelflocken, welche die fertilen Hyphen enthalten. Farbe der Kultur gegen das Licht olivgrün. Geruch säuerlich. Unterseite olivgrün. Desgl. nach 14 Tagen: eine geschlossene schwarze Decke, welche überdeckt wird von einer flachwolligen, leichtbraunen Schicht, bestehend aus zahlreichen kleinen, an den Trägern sich entwickelnden Sporentröpfchen. Unterseite grauschwarz.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine schwarze Decke mit flachwolligem Ueberwuchs von zahlreichen, Sporenköpfchen bildenden Konidienträgern.

Auf Kirsch-Agar: wie vorige.

Auf Möhre: das Stück ganz bewachsen von einer schwarzen, glatten, feuchten Haut, stellenweise mit Konidienträgern.

Auf Kartoffelstück: wie vorige.

Lateinische Beschreibung.

Conidiophoris 4—6 $\mu$ latis, pediculo brunneo praeditis. Acumine ramatis sicut penicillium. Ramulis extremis 10—16 $\mu$ longis, 2 $\mu$ latis. Conidiis

consistentibus una cella, ellipsoideis, variae crassitudinis, (4—12) × (2—4,7) μ — plerumque (5,3—6,3) × (2,7—3,3) μ —, eguttulatis, attamen vacuola praeditis vel ambigua protoplasma continentibus.

### *Gliocladium cibotii* nov. spec.

Diesen Pilz erhielt das C. B. S. von Frl. M. F. Habekotté in Delft, wo er von den Haaren des Baumfarnes *Cibotium Schiedei* isoliert wurde. Bei üppigem Wachstum bildet er gern Hyphenbündel, von denen die Konidienträger allseitig abgehen. Diese Konidienträger können einfach sein, in den meisten Fällen jedoch sind sie gabelförmig verzweigt mit 2 oder 3 Aestchen. Dadurch nähert sich der Pilz der Gattung *Verticillium* und es fällt manchmal schwer, beim Studium der systematischen Stellung für dergleiche Organismen die richtige Wahl zu treffen. Es sei daher gestattet auf das Verhältnis zwischen *Gliocladium* und *Verticillium* an dieser Stelle etwas näher einzugehen.

In der ursprünglichen Diagnose von *Gliocladium* Corda entstehen die Konidien nicht in Ketten, sondern einzeln nacheinander, schliesslich durch Schleim zu einem Köpfchen verklebt, im Gegensatz zu *Penicillium*, wo die Sporen in Ketten erzeugt werden, welche nicht verschleimen. Später wurden auch Arten beschrieben, welche die Konidien in Ketten bildeten, die sich dann schliesslich ebenfalls zu verschleimten Köpfchen zusammenballten. Die Verzweigung der Konidienträger bei *Gliocladium* soll mehr oder weniger penicilliumartig sein, jedenfalls nicht in mehreren Wirteln übereinander wie bei *Verticillium*, sondern höchstens gabelförmig mit 2—3 Endästchen. Nun können auch Arten aus dem Formenkreise der Verticillien eine ähnliche einfache Verzweigung der Konidienträger aufweisen, welche auch auf den besten Nährböden nur selten darüber hinaus geht, sodass man im Zweifel sein kann, ob ein *Gliocladium* oder ein *Verticillium* vorliegt. In dem Falle kann dann die Anwesenheit von braunen Chlamydosporen entscheidend sein für die systematische Stellung eines solchen Pilzes. Sind diese nämlich im Myzel vorhanden, so fallen die betreffenden Organismen in den Formenbereich von *Verticillium dahliae* Klebahn. Von van Beyma (4) wurden dergleiche Formen beschrieben als *Verticillium dahliae* Klebahn forma *restrictum* und *Verticillium dahliae* Klebahn forma *zonatum*. Als Zwischenform zwischen *Verticillium* und *Gliocladium* sei *Gliocladium roseum* Bainier genannt. Foëx (7) fand die *Verticillium*-Form dieses Pilzes auf Kartoffeln; hier kann sie eine Fäule verursachen wobei im Gewebe zahlreiche Sklerotien entstehen. Nachdem Foëx den Pilz dem C. B. S. zugeschickt hatte, wurde er von van Beyma als ein richtiges *Verticillium* betrachtet und unter dem Namen *Verticillium Foëxii* nov. spec. beschrieben(3). Die *Penicillium*-Form war seinerzeit von Link *Penicillium roseum* genannt worden, wurde jedoch später von Bainier (1) auf Grund der in feuchter Umgebung sich bildenden Sporenköpfchen zur Gattung *Gliocladium* gebracht. Da beide Formen nebeneinander

vorkommen können, ist es schwer zu entscheiden, wohin der Pilz eigentlich gehört; der ältere Name *Gliocladium* mag daher vorläufig den Vorzug haben. Abbildungen beider Formen, der *Acrostalagmus-* (= *Verticillium*) Form und der *Penicillium-* Form findet man bei THOM in seiner Monographie über *Penicillium* (10).

Wenn wir nun bei unserem Pilze die hier erwähnten Erwägungen in Betracht ziehen, kann von einer eigentlichen *Verticillium*-Form wohl nicht die Rede sein, da die Zahl der Aestchen 3 nicht übersteigt, die Wirtel also möglichst einfach sind und mehrfach quirliche Verzweigung nicht auftritt. Auch fehlen schliesslich braune Chlamydosporen. Daher kann kaum ein Zweifel darüber bestehen, dass der Pilz am besten zu *Gliocladium* gestellt wird.

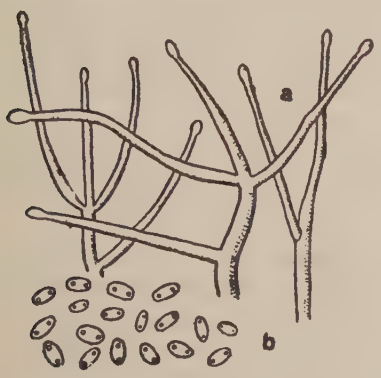


Fig. 4. *Gliocladium cibotii.*
*a.* Konidienträger. Vergr. 750 ×.
*b.* Konidien. Vergr. 750 ×.

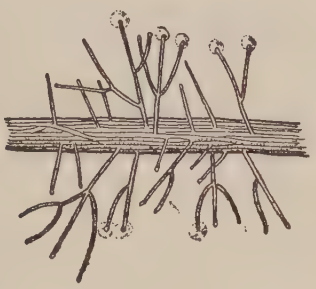

Fig. 5. *Gliocladium cibotii.*
Hyphenbündel mit Konidienträgern. Vergr. 245 ×.

Auf allen Nährböden wächst er anfangs mit einem weissen, etwas wolligen Myzel, das alsbald in den tieferen Schichten eine dunkelbraune bis schwarze Farbe annimmt. Die Konidienbildung war anfangs eine sehr üppige, die Sporenköpfchen flossen zu unzähligen Tröpfchen zusammen. Im Verlauf der Weiterzüchtung hat jedoch diese Konidienbildung etwas nachgelassen, sowie auch die anfängliche Neigung zur Erzeugung von aufstehenden Hyphenbündeln.

Die Beschreibung des Pilzes lautet wie folgt:

*Gliocladium cibotii* nov. spec.

*Hyphen* zart, 2—3 $\mu$ dick, leicht gefärbt, gern zu Hyphenbündeln zusammentretend, welche dann eine grünbraune bis braunschwarze Farbe aufweisen.

*Konidienträger* von den Hyphenbündeln in grosser Zahl abgehend, 80—100 $\mu$ lang und 2—3 $\mu$ dick, wenig verzweigt, meist gabelförmig mit 2—3 Sterigmen.

*Sterigmen* gerade, pfriemenförmig, 20—40 $\mu$ lang, an der Basis 2 $\mu$ breit, meist 2—3 an der Zahl, opponiert oder wirtelig stehend.

*Konidien* massenhaft, zu falschen Köpfchen verklebt, hyalin, einzellig, ellipsoidisch, (4—6) × (2—2,7) $\mu$, meist (4—4,7) × (2,3—2,7) $\mu$, mit zwei Oeltröpfchen.
*Hab.* Isoliert aus den Haaren des Baumfarnes *Cibotium Schiedei* (Frl. M. F. Habekotté, Delft).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 1 Monat: Kolonie 7 cm im Durchmesser, im Zentrum mit mehreren, bis 1 cm hohen, weissen Hyphenbündeln. Um das Zentrum herum eine grauschwarze filzige Decke mit mehreren tiefen, radiären Falten; hier findet üppige Konidienbildung statt. Der Rand besteht aus einer breiten Zone von dünnen, farblosen, sterilen, bis 1 cm hohen Hyphenbündeln. Geruch schwach würzig. Unterseite im Zentrum schwarz, sonst blass gelblich.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: unten im Röhrchen eine weisse, filzige Decke, ohne oder mit spärlicher Konidienentwicklung.

Auf Kirsch-Agar: die Agar-Oberfläche überdeckt von kleinen, zahlreichen, weisslichen Sporentröpfchen. Stellenweise erheben sich bis 3 mm hohe, in Büscheln beisammen stehende Hyphenbündel, welche zahlreiche Konidienträger erzeugen.

Auf Möhre: das Stück ganz bewachsen von einer filzigen, weissen Decke, von welcher zahlreiche Hyphenbündel sich erheben.

Auf Kartoffelstück: wie vorige.

Auf Kartoffel-Agar: das Wachstum stimmt mit dem auf Kirsch-Agar überein, üppige Konidienbildung.

Auf Haferflocken-Agar: wie vorige, nur bildet sich am Glase ein schwarzer Rand, der bei Kartoffel-Agar fehlt.

Lateinische Beschreibung.

Hyphis saepe conjunctis densis fascibus atrobrunneis. Conidiophoris permultis, ex hyphorum fascibus exeuntibus, 80—100 $\mu$ longis et 2—3 $\mu$ crassis, plerumque furcae in modo ramatis, 2—3 sterigmis praeditis, rectis, subuliformibus, 20—40 $\mu$ longis, basei 2 $\mu$ latis, oppositis vel vorticis in modum positis. Conidiis numerosissimis, in caespitulis cohaerentibus, hyalinis continuis ellipsoideis, (4—6) × (2—3,7) $\mu$, plerumque (4—4,7) × (2,3—2,7) $\mu$, cum duobus guttulis oleaginis.

## *Margarinomyces mutabilis* nov. spec.

Das C. B. S. erhielt diesen Pilz unter No 1661 von Dr. H. Windisch (München) der ihn im Sommer 1942 aus einer zur biologischen Untersuchung eingesandten Flusswasserprobe isolierte.

Auf Agar-Nährböden wächst er mit zottigem Myzel, bestehend aus Hyphenbündeln, welche mit ihren zahlreichen Konidienträgern die Konidien in grossen Mengen abschnüren. Dagegen ist das Wachstum auf Kartoffelstück und Möhre schlecht; hier entstehen nur glatte, submerse Häute ohne Konidienentwicklung. Anfänglich ist

die Pilzdecke auf Bierwürze-Agar weiss, wird dann innerhalb 4 Wochen durch massenhafte Bildung, vom Zentrum aus, von endständigen und gewöhnlichen Konidien schwarz, mit einem Ueberwuchs von weissem Myzel mit kurzen, verfilzten Hyphenbündeln.


Fig. 6. *Margarinomyces mutabilis.*
*a.* Konidienträger aus Agarkulturen. Vergr. 750 ×.
*b.* Konidienträger aus feuchte Kammerkultur. Vergr. 750 ×.
*c.* Konidien. Vergr. 750 ×.

Die endständigen Konidien sind meist einzellig. Sie entstehen am Ende eines Fadens oder seitlich an demselben und schwimmen im mikroskopischen Präparat umher als subglobose oder ellipsoidische, dickwandige, braune Zellen. Mitunter bilden sich auch zwei der-

Fig. 7. *Margarinomyces mutabilis.*
Endständige Konidien. Vergr. 750 ×.

gleicher Zellen nacheinander, sie werden dann durch eine Querwand getrennt. Ein solches Gebilde stellt, frei geworden, eine zweizellige Konidie dar; diese kommen jedoch verhältnismässig selten vor. Eine Verwechslung mit *Phialophora mustea* Neergaard, die ebenfalls endständige Konidien in grosser Zahl erzeugt, ist ausgeschlossen, da letztgenannte auf allen Nährböden sofort eine schwarze Haut bildet, weil hier die dunkelfarbigen endständigen Konidien gleich vom Anfang an sich entwickeln.

Die Erzeugung von gewöhnlichen Konidien geschieht an mehr oder weniger flaschenförmigen, unverzweigten oder verzweigten Trägern, die entweder einzeln an den Hyphen entlang oder büschelartig beisammen stehen. Ein ganz anderes Bild ergibt die Kultur des Pilzes in einer feuchten Kammer. Dann werden, ähnlich wie bei *Pullularia pullulans* die Konidien in schneller Folge massenhaft an kleinen Vorsprüngen der fertilen Hyphen abgeschnürt, wonach sie sich zu Konidienköpfchen zusammenballen. Durch die Bildung der endständigen Konidien unterscheidet der Pilz sich von allen übrigen Arten dieser und der nahestehender Gattung *Phialophora* (5), weshalb die Beschreibung als neue Art unten folgt.

*Margarinomyces mutabilis* nov. spec.

*Pilzdecke* anfangs weiss, zottig durch aufstehende Hyphenbündel, später infolge der Bildung von endständigen Konidien schwärzlich.

*Konidienträger* mehr oder weniger flaschen- bis birnförmig, selten verzweigt, 10—20 $\mu$ lang, an der breitesten Stelle 2,7—4 $\mu$ dick, einzeln oder büschelweise an den fertilen Hyphen entlangstehend, die Konidien einzeln abschnürend.

*Konidien* massenhaft, einzellig, hyalin, ellipsoidisch, mit zwei Oeltröpfchen, (4,3—6,7) × (1,7—3) $\mu$, meist (4,7—6) × (1,7—2,3) $\mu$, in feuchter Umgebung auch unmittelbar an kleinen Ausstülpungen der Hyphen entstehend, zu kleinen Köpfchen verklebend.

*Endständige Konidien* in älteren Kulturen massenhaft, dickwandig, braun, glatt, fast undurchsichtig, oft mit 1—2 Vakuolen im Innern, meist einzellig, seltener zweizellig, akrogen oder pleurogen an den Hyphen entstehend, (6,3—10,7) × (5,3—6) $\mu$ gross.

*Hab.* Aus einer Flusswasserprobe (H. Windisch, München).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 30 Tagen: Langsam sich ausbreitende Kolonien, 5 cm im Durchmesser, bestehend aus einer zähen Haut, überwachsen von zahlreichen kurzen, weissen Hyphenbündeln, welche die dunklere konidienbildende Schicht bedecken. Im Zentrum ein Polster aus grauen und weissen Hyphenbündeln, fast 1 cm hoch. Schwacher Geruch. Unterseite im Zentrum schwärzlich, sonst etwa 247.

Auf Röhrchen nach 10 Tagen:

Auf Bierwürze-Agar: eine zottige, hellgelbbraune Kolonie, bestehend aus 1—3 mm hohen Hyphenbündeln, von denen die Konidienträger in grosser Zahl abgehen.

Auf Kirsch-Agar: wie vorige.

Auf Möhre: nur eine submerse, blassgelbe Haut ohne Konidienentwicklung.

Auf Kartoffelstück: nur eine dünne, glatte, feuchte Haut ohne Konidienentwicklung.

Auf Kartoffel-Agar: eine zottige, gelbbraune Kolonie, bestehend aus 1—3 mm hohen Hyphenbündeln. Der Agar etwas gelb gefärbt.

Auf Haferflocken-Agar: eine submerse, farblose Haut mit einzelnen, lockeren, farblosen Hyphenbündeln.

Lateinische Beschreibung.

Conidiophoris plus vel minus ampullaceis vel piriformibus, rariter ramatis, 10—20 $\mu$ longis, latissima parte 2,7—4 $\mu$ crassis, singulis vel fascinium in modum in hyphis fertilibus positis, conidia singula ferentibus. Conidiis numerosissimis, una cella consistentibus, hyalinis ellipsoideis, duabus oleaginis guttulis praeditis, (4,3—6,7) × (1,7—3) $\mu$, plerumque (4,7—6) × (1,7—2,3) $\mu$. Conidiis cacumine numerosissimis in vetustioribus culturis; crassis parietibus, brunneis, levibus, plerumque continuis, (6,3—10,7) × (5,3—6) $\mu$.

## *Scopulariopsis capsici* nov. spec.

Aus dem Laboratorium der „Specerijen-Malerij De Körver" in

Boxmeer (Niederl.) erhielten wir die Kultur einer *Scopulariopsis*, isoliert von Paprika (*Capsicum annuum* L.). Dieser Pilz besitzt ein langsames Wachstum auf allen Nährböden und bildet meist filzige, graubraune oder dunkelbraune, faltige Häute, von strähnigen, verfilzten, fertilen Hyphen überwachsen. Die Hyphen sind braun gefärbt, haben einen körnigen Inhalt und ein unregelmässiges Lumen. Sie treten gern zu dicken Hyphenbündeln zusammen, von welchen dann die Konidienträger dichtgedrängt allseitig abgehen. Die meisten Nährböden werden von dem Pilze dunkelbraun verfärbt. Die Beschreibung des Pilzes lautet folgendermaassen:

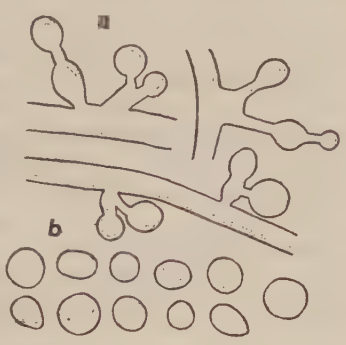


Fig. 8. *Scopulariopsis capsici*.
*a*. Konidienträger. Vergr. 750 ×.
*b*. Konidien. Vergr. 750 ×.

*Scopulariopsis capsici* nov. spec,

*Konidienträger* wenig verzweigt, meist aus einzelnen Sterigmen bestehend.

*Sterigmen* von unregelmässiger Gestalt: spielkegelförmig, gerade oder schwach gekrümmt, in der Mitte etwas aufgetrieben; oder breit ausladend, am Scheitel mehrere Konidien zugleich abschnürend; oder auch tonnenförmig, den Hyphen breit aufsitzend, 10—20 $\mu$ lang, an der breitesten Stelle 2—5 $\mu$ breit.

*Konidien* entweder eiförmig, d.h. an ihrem unteren Ende zugespitzt und am anderen Ende abgerundet oder subglobos, glatt, einzeln hyalin bis leicht gefärbt, in grossen Massen braun, oft mit kleinen Tröpfchen im Innern, in kurzen, leicht auseinander fallenden Ketten entstehend, 6—8 $\mu$ gross.

*Perithezien* wurden nicht beobachtet.

*Hab.* Isoliert von *Capsicum annuum* L. (Specerijen-Malerij De Körver, Boxmeer).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 8 Tagen: Langsam wachsend, Kolonie 1,5 × 1 cm im Durchmesser, wollig durch aufstehende, stark verfilzte Hyphensträhnen, welche die Konidien in grossen Massen erzeugen. Die Konidienfarbe ist graubraun. Um den Kolonien herum ein farbloser Saum, 0,5 mm breit. Kein Geruch. Unterseite etwa 268.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: unten im Röhrchen eine kleine, flachwollige Kolonie mit zahlreichen Konidienträgern an den Hyphen, graubräunlich. Am Impfstück weisse, filzige Hyphenbündel. Agar braun gefärbt.

Auf Kirsch-Agar: am Impfstück entlang zahlreiche, hell-

graubräunliche, konidienbildende Hyphenbündel mit farblosen, kleinen Wassertröpfchen. Agar nicht gefärbt.

Auf Möhre: das Stück ganz bewachsen von einer glatten, feuchten, schwarzen Haut, von der sich zahlreiche braune, 1 mm hohe Hyphenbündel erheben.

Auf Kartoffelstück: das Stück ganz bewachsen von einer glatten, trockenen, schwarzen Haut mit vereinzelten, aufstehenden, schwarzen Hyphenbündeln und grösseren, hellbraungrauen Konidienpolstern.

Auf Reis: 2 cm tief gewachsen, die Körner überdeckt von grauen Konidienpolstern mit bräunlichem Stiche.

Auf Haferflocken-Agar: eine submerse, schwarzgrüne Haut mit spärlicher Konidienbildung.

Lateinische Beschreibung.

Conidiophoris rariter ramatis, plerumque consistentibus ex singulis sterigmis. Sterigmis irregulariter formatis, rectis vel leviter curvatis, medio quodammodo intumescentibus 10—20 $\mu$ longis, latissima parte 3—5 $\mu$ crassis. Conidiis ovoideis vel subglobosis, hyalinis vel tenuiter coloratis, levibus; si numerosis brunneis 6—8 $\mu$ crassis.

## *Scopulariopsis croci* nov. spec.

Das „Laboratorium voor Bloembollenonderzoek" in Lisse (Niederl.) schickte dem C. B. S. die Kultur einer *Scopulariopsis*, die von einer Crocus isoliert worden war. Auf allen Nährböden wächst der Pilz nur langsam an unter Bildung unebener Decken, welche eine puderige, braunschwarze Konidienschicht tragen, mit spärlichem Luftmyzel und einzelnen farblosen Wassertröpfchen. Die fertilen Hyphen schliessen sich gern zu Hyphenbündeln zusammen, von denen dann allseitig die Konidienträger abgehen. Letztere sind meist unverzweigt. Ihre Länge überschreitet meist nicht 15 $\mu$, die Breite beträgt etwa 3—4 $\mu$. Die Konidien werden in kurzen Ketten abgeschnürt, sie sind für eine *Scopulariopsis* ziemlich klein, nämlich etwa 4 $\mu$ im Durchmesser. Anfangs sind sie zitronenförmig, hyalin und glatt, im Alter runden sie sich ab und werden rauh oder stachelig und braun. Die Beschreibung des Pilzes lautet:

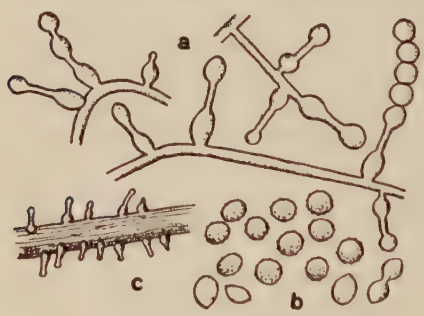


Fig. 9. *Scopulariopsis croci*.

*a*. Konidienträger. Vergr. 750 ×.
*b*. Konidien. Vergr. 750 ×.
*c*. Hyphenbündel mit Konidienträgern. Vergr. 245 ×.

*Scopulariopsis croci* nov. spec.

*Konidienträger* meist unverzweigt, seltener verzweigt, einzeln an den fertilen Hyphen entlang stehend.
*Sterigmen* etwa von Spielkegelgestalt, 13—15 $\mu$ lang, an der breitesten Stelle 3—4 $\mu$ dick, meist einzeln stehend.
*Konidien* anfangs zitronenförmig, hyalin und glatt, später sich abrundend und rauh oder stachelig und braun werdend, 4—4,7 $\mu$ im Durchmesser.
*Perithezien* wurden nicht beobachtet.
*Hab.* Isoliert von einer Crocus-Varietät „Queen of the Blues", (Lab. voor Bloembollenonderzoek, Lisse, Niederl.).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: Langsam wachsend, Kolonien 1 cm im Durchmesser, flach hügelig, von etwas wolligem Myzel überwachsen, im Zentrum braungrau, n.d. Rande hin heller. Rand filzig, weiss, 0,5 mm, in einen farblosen Saum übergehend. Kein Geruch. Unterseite mit 2 Zonen. Farbe etwa 297, im Zentrum oft etwas dunkler.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine unregelmässig gewölbte und von kurzem Filz uberwachsene Decke, hellbraungrau. Rückseite dunkelbraun.

Auf Kirsch-Agar: wenig angewachsen, einige kleine, farblose, häutige Bildungen ohne Konidien.

Auf Möhre: das Stück ganz bewachsen von einer Haut mit rundlichen Auswüchsen, welche zum Teil farblos, zum Teil schwarz sind. An den braunschwarzen Stellen Konidienbildung.

Auf Kartoffelstück: das Stück ganz bewachsen von einer hellgraubraunen, glatten, feuchten Haut mit rundlichen Auswüchsen, worauf sich stellenweise braungraue Konidienpolster entwickelt haben. Das Stück etwas braungelb verfärbt.

Auf Reis: 1,5 cm tief gewachsen, an den Körnern braungraue Konidienpolster.

Auf Haferflocken-Agar: eine üppige Entwicklung von kurzen, filzigen Hyphenbündeln, hellgrau, mit reichlicher Konidienbildung und zahlreichen, farblosen Wassertröpfchen. Rückseite farblos.

Lateinische Beschreibung.

Conidiophoris non ramatis. Sterigmis conoideis 13—15 $\mu$ longis, latis ima parte 3—4 $\mu$ crassis. Conidiis primo citreniformibus, levibus, hyalinis, postea se conglobantibus, rudibus vel spineis atque brunneis esse incipientibus 4—4,7 $\mu$ crassis.

## *Penicillium australicum* (Olsen-Sopp) emend. van Beyma

Schon mehrere Jahre befindet sich in der Sammlung des C. B. S. ein *Penicillium*, eingesandt von Dr. H. Zach im Juli 1928, unter

der Bezeichnung *Penicillium australicum* (Kap Labor) Hann. Es fehlt also ein Autorname, denn Kap Labor ist das Laboratorium von JOHANN OLSEN-SOPP in Mjösen (Norwegen), während Hann., eine Abkürzung von Hannover, sich auf das Laboratorium von C. WEHMER bezieht. Wahrscheinlich hat ZACH das *Penicillium* von WEHMER bezogen und es seinerzeit dem C. B. S. zugeschickt. Auch in einer alten Pilzliste von 1924 von Prof. Dr. PRIBRAM's mikrobiologische Sammlung (vorm. KRAL's bakteriologisches Museum) in Wien ist unser *Penicillium* als *Penicillium australicum* (Kap) aufgeführt. Da es gerade in der letzten Zeit wiederholt von verschiedenen Substraten pflanzlicher Herkunft isoliert wurde, ist damit auch die Frage nach dem Autor wieder akut geworden. Ein Schreiben unserseits an das Kap-Laboratorium blieb unbeantwortet. Herr H. ROBAK in Bergen (Norwegen) hatte auf unsere Anfrage die Freundlichkeit mitzuteilen, dass OLSEN-SOPP schon vor einigen Jahren verstorben und das Kap-Laboratorium nach aller Wahrscheinlichkeit aufgehoben worden ist.

Es kann jedoch kaum ein Zweifel darüber bestehen, dass *Penicillium australicum* von OLSEN-SOPP isoliert wurde, wie die Bezeichnung Kap andeutet, wahrscheinlich ist er niemals dazu gekommen, eine Beschreibung des Pilzes zu veröffentlichen. THOM erwähnt in seiner Monographie (10) den Namen bloss im Inhaltsverzeichnis. Da *Penicillium australicum* jedoch eine gute Art darstellt, die auch hin und wieder mal im C. B. S. angefragt wird, ist es u.E. unbedingt notwendig, eine Beschreibung dieses Pilzes zu geben. Um unnötige Verwirrung zu vermeiden, halten wir es für angebracht, den Namen *australicum* beizubehalten und aus obengenannten Gründen OLSEN-SOPP als den mutmasslichen Entdecker aufzuführen. Die Herkunft des Pilzes wird wohl unaufgeklärt bleiben.

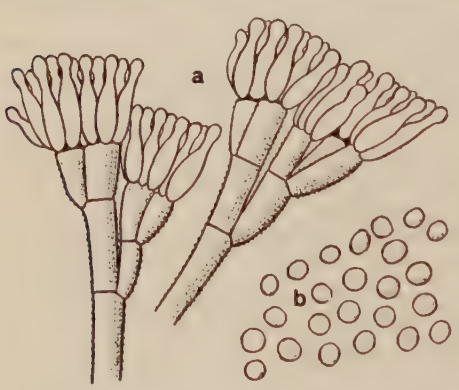


Fig. 10. *Penicillium australicum*.
*a*. Konidienträger. Vergr. 750 ×.
*b*. Konidien. Vergr. 750 ×.

*Penicillium australicum* gehört zu den schnellwüchsigen Penicillien. Die anfangs blaugrüne Decke färbt sich alsbald mehr reingrün und es treten Zonen auf. Typisch für den Pilz sind die ziemlich grossen, subglobosen Konidien, der Geruch nach Kartoffeln und der orangefarbene Saum auf Bierwürze-Agar um jede Kolonie herum. Die Beschreibung des Pilzes lautet folgendermaassen:

## *Penicillium australicum* (Olsen-Sopp) emend. van Beyma.

*Rasen* stark puderig durch üppige Konidienbildung, 372—366—348. Deutlicher Schimmelgeruch, etwa wie Kartoffeln im Keller. Unterseite gelb. Um den Kolonien herum ein orangefarbener Saum, 1 mm breit, auf Bierwürze-Agar.

*Konidienträger* etwa 4 $\mu$ dick, rauh punktiert, verzweigt, mit 2—3 Sekundärästen, welche ihrerseits wieder je 2—3 Aestchen aufweisen, aus denen dann schliesslich die Sterigmen hervorgehen. Letzte Aeste 10—13 $\mu$ lang und 4 $\mu$ dick, je 3—5 Sterigmen tragend.

*Sterigmen* zahlreich, dicht gedrängt stehend, flaschenförmig, 13—15 $\mu$ lang und 3,3—4 $\mu$ dick.

*Konidien* subglobos bis kugelig, glatt, meist (4—4,7) × (3 — 4) $\mu$ gross.

*Hab.* Wiederholt isoliert von Pflanzenmaterial und aus der Erde (C. B. S., Baarn).

*Reinkulturen:*

Auf Bierwürze-Agar in einer Petrischale nach 5 Tagen: Kolonien flachwollig bis puderig, 372—366 oder etwas grüner, mit schmalem, weissem Rande. Deutlicher Schimmelgeruch. Um die Kolonien herum ein farbloser Saum, 2 mm breit. Kolonien im Zentrum etwas erhöht, körnig. Undeutliche Zonenbildung. Unterseite gelb, 221—216. Desgl. nach 10 Tagen: Decke puderig, fast ohne Rand, im Zentrum meist mit Myzelbüscheln, Farbe zwischen 347 und 342, nach dem Rande mehr 372. Grössere Kolonien mit radiären Falten. Rand körnig, weiss bis grünlich weiss, 1 mm. Saum 1—2 mm, orangefarben. Deutliche Zonen. Starker Geruch nach Kartoffeln. Unterseite im Zentrum 156—186, Rand 182.

Auf Röhrchen nach 7 Tagen:

Auf Bierwürze-Agar: Kolonien stark puderig bis puderig-wollig mit schmalem, weissem Rande. Rückseite 152—177—291.

Auf Kirsch-Agar: Kolonien puderig, blaugrün, etwas grüner wie 373. Unten im Röhrchen weisser Rand, 1,5 mm.

Auf Möhre: Ganz bewachsen, puderig mit vielen farblosen Wassertropfen, 368—348, oder etwas dunkler. Rand am Glase gelb, 221.

Auf Kartoffelstück: Fast ganz bewachsen, flachwollige bis filzige Haut, blaugrün, oben Farbe zwischen 368 und 373, oder stellenweise 367, ganz unten 366. Farblose Wassertröpfchen. Rand am Glase 221.

Auf Raulin: Decke flachwollig mit einigen Querfalten, oben weiss, unten 396.

Auf Reis: 1 cm tief gewachsen, oben blaugrün, 366—367—368. Der Reis stark gelb, 216—156. Am Glase 396.

Auf Haferflocken-Agar: mässiges Wachstum. Decke flach-puderig, 372—373.

## Lateinische Beschreibung.

Conidiophoris plus minus 4 $\mu$ crassis, verruculosis, ramis praeditis, duos vel tres minores ramos gerentibus, duobus vel tribus ramiculis praeditis, quae 10—13 $\mu$ longa et 4 $\mu$ crassa sunt, quaeque singula 3—5 sterigmata gerunt. Sterigmatibus numerosis, densis, ampullaceis, 13—15 $\mu$ longis et 3,3—4 $\mu$ crassis. Conidiis subglobosis vel globosis, levibus plerumque (4—4,7) × (3 — 4) $\mu$ crassis, catenas firmas formantibus, odore mucido ethereo.

## Zusammenfassung.

Es wurden folgende Pilze im Centraalbureau voor Schimmelcultures zu Baarn neu beschrieben: *Chaetomium minimum*, *Piptocephalis macrospora*, *Scopularia corsicana*, *Gliocladium cibotii*, *Margarinomyces mutabilis*, *Scopulariopsis capsici*, *Scopulariopsis croci*, *Penicillium australicum*.

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(From the Rijks Instituut voor de Volksgezondheid, Utrecht).

# ON THE PREPARATION OF VACCINE AGAINST TYPHUS FEVER AND THE EXPERIENCES GATHERED THEREWITH [1])

by

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The preparation of the vaccine against typhus fever does not differ in principle from the method along which the better known bacterial vaccines as those against typhoid fever, cholera, wooping cough are prepared. In fact in such cases it is aimed at to obtain a suspension of disease germs killed of by means of phenol or formaldehyde of such density, that a sufficient immunity may be provoked after a subcutaneous injection in man. Nevertheless for the preparation of vaccine quite another line must be taken up in case of typhus fever than for the above mentioned bacterial vaccines. As it is the causal agents of typhus fever, the Rickettsiae, do not belong to the bacteria, but may rather be reckoned among the viruses. They do not multiply (as far as we know up till now) on artificial media, but need for their growth the presence of living tissue. So new methods for the preparation of vaccine have been tried out with more or less success.

I will not go further into the various methods which have been worked out and which I have already discussed elsewhere (14), with the exception of a single one. Here a method of production of vaccine is concerned which up till now has proved very successful in practice, but which may not be applied in the Netherlands, the reason of which will be explained further down. A Polish investigator WEIGL (16 has contrived the nearest approach to the solution of the problem of the preparation of vaccine against typhus fever, which as a matter of fact is still in the experimental phase. He used as a culture medium for the multiplication of the Rickettsiae the lice (15). Nowadays it may be deemed well known that lice transmit the typhus fever, at least the disease occurring in East Europe. This insect has this property thanks to the fact that once infected with Rickettsiae these agents of disease can multiply at a quick rate in its body. They invade the endothelial cells of stomach and guts, where they multiply in such amounts that these cells will be filled

[1]) Paper read in the meetings of November 21th, 1942 and November 15th, 1943 of the Nederlandsche Vereeniging voor Microbiologie.

with them next to bursting. Finally the endothelial cells will burst and their contents will be released in the lumen of the gut. At such a moment the faeces will consist chiefly out of Rickettsiae.

This process, which occurs under natural conditions after the louse has sucked blood of a patient of typhus fever some days before or during the febrile stage (6) where it gets infected with Rickettsiae, WEIGL imitates artificially. In this end he infects the louse per anum with Rickettsiae containing material. The multiplication of Rickettsiae occurs then along the same line as described above, so that after a period of 5 to 8 days the lumen of the gut of these insects teems with Rickettsiae, out of which vaccine may be prepared.

Although this vaccine has been prepared on a large scale and its value has been established irrefutably, the above mode of preparation of vaccine cannot be applied in the Netherlands. The lice infected with Rickettsiae need as a matter of fact a period of 5—8 days to bring the disease germs to full development. During this period these strongly infective animals feed on human blood. Obviously persons, who consent to this treatment must be completely immune against typhus fever. Along with the sucking of blood by the lice the Rickettsiae are easily transmitted to man. In countries such as Poland, where this disease is of very frequent occurrence the finding of such immune persons does not offer difficulties. In a country free from typhus fever as which he Netherlands may fortunately still be considered, such persons are nor to be met with and thus this method for the preparation of vaccine cannot be applied.

As soon as it had been decided that the preparation of vaccine against typhus fever would be taken on hand, which under present conditions was assuredly indicated, some other method had to be followed. The chosing of a method out of the various existing ones was facilitated by the fact, that a method existed, which next to being relatively simple, had produced already good results. In this method, such as it has been described by COX (3, 4), the developing fertile hen's egg is used as a nutrient medium for the growth of Rickettsiae. This method for the preparation of vaccine is frequently applied. In the ,,Staatliches Institut für Experimentelle Therapie" at Frankfurt this method has been applied successfully by OTTO and WOHLRAB (12). GILDEMEISTER and HAAGEN (9) applied it as well, whilst TCHANG and MATHEWS (18) judge this method very favourably in a communication from PEIPIN.

But before we could take the preparation of vaccine in hand, we needed to dispose of strains of typhus fever. These were not available in the Netherlands. Prof. OTTO in Frankfurt had the kindness to hand over some to me. Along with the taking over of these strains, which took place in Frankfurt, I had the opportunity to become acquainted with the preparation of vaccine, an opportunity of which I made a grateful use.

By these means I obtained three strains of typhus fever, *viz.*, 2 originating from East European cases of typhus fever respec-

tively from Cracow and Warsaw, thus belonging to the type „Prowazek" and a strain originating from America which is to be reckoned to the type „Mooser".

For the preparation of vaccine the Rickettsiae are killed of with phenol or formaldehyde and only the Prowazeki strains can be used for this end. The less virulent and thus less dangerous Mooser strains cannot serve here as these Rickettsiae in living condition may induce sufficient immunity against the Prowazeki strains but not when dead [1]). And as the cases of typhus fever occurring in East Europe are caused by this race and as from those regions the danger for the Netherlands is most imminent, the Prowazeki strain had to be used for the preparation of vaccine.

These strains are kept active by animal passage, guinea pigs being the most suitable experimental animals (11). They are generally very susceptible for this infection. Usually it will not result in their death, but after the inoculation they will produce a typical fever curve. In the last two years we experienced that the susceptibility of the guinea pigs may strongly vary and depends on their batch of origin. A seasonal influence was hardly to be noted; we are of the opinion that it might rather be ascribed to a difference in susceptibility in the different breeds of guinea pigs. This was all the more probable as in the cultivation of the Mooser strains on mice we have been struck by a phenomenon of the same nature. The susceptibility of various breeds of mice appeared to vary from 0 % to 100 %. It is certainly noteworthy that information reached us from Tunesia about a 100 % susceptibility of the mice for Mooser strains, Frankfurt on the other hand mentions a susceptibility of 60 %, whilst here an average of merely 20 % was reached. Note has to be taken of the fact, that in Frankfurt and here a same Mooser strain has been worked with.

From a correspondence on this subject with Dr. BICKHARDT, Head of the Department for Typhus fever of the Staatliches Institut für Experimentelle Therapie at Frankfurt it appeared that the same Mooser strains, which WOHLRAB had taken over to Poland, remained there ineffective for mice. It appears to us, according to our limited experience, not improbable that hereditary factors are to be made responsible for this discrepancy in results. Under more normal conditions when we may dispose over more experimental animals we hope to gather more exact data.

For the preparation of the vaccine first of all the Rickettsiae have to be transmitted from the guinea pig into the fertile egg. As has been stated already guinea pigs produce a typical fever curve, which takes the following course: starting from the moment of inoculation the temperature initially will not offer anything notable.

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[1]) The preparation of the so called living vaccines in which the Rickettsiae have not been killed of has not been taken up by me, as it involves always dangers. I refer to a publication of BIRAUD (1) wherein for countries free from epidemics preference is given to „dead vaccines".

Now and then on the third or fourth day a slight raise in temperature may occur, which will fall to the norm again; suddenly on the sixth or seventh day it will rise steeply up till over 40° C. The temperature remains on this height during six to ten days and will then sink lytically. For the examination of the eggs (and moreover for the transmitting interperitoneally of the strain on the next guinea pig in view of the conservation of the strain) the infected guinea pig is killed on the fourth day of fever. It is supposed that at such a moment the Rickettsiae occur in greatest density in the brain substance. The brains are removed sterilly and brought in suspension in saline.

For the culture of Rickettsiae fertile eggs are used, which have been kept in the incubator at 39° C. at least five and at most eight days. During this period the eggs are viewed dayly, dead ones being discarded and the others turned over 180°.

Before the inoculation with Rickettsiae the eggs are washed with alcohol and then put up vertically with the air space in top. The area of the shell which covers the air space is painted with tincture of iodine. In its centre a small opening is made with a firm injection needle and the needle of the syringe is passed through. The syringe contains the above mentioned brain substance. The needle itself is pushed about 4 cm vertically into the egg and then slowly 0.5 ml of the content of the syringe is injected. The needle is drawn out of the egg and the opening of the shell is covered with paraffine.

As within 24 hours after the inoculation of the egg a great mortality occurred, it was tried to prevent this. Therefore first of all the dose of Rickettsiae of 0.5 ml as it was in use in Frankfurt, was brought down gradually. Obviously the percentage of the eggs which would take the infection might not decrease or at least as slightly as possible. Finally the amount of 0.2 ml was established, which allowed of a markedly lower „mortality within 24 hours", whilst the getting hold of the infection had not decreased worth mentioning.

In the first meeting in 1942 another factor was mentioned as possibly causing the early death of the embryos, *viz.*, the inoculation of the eggs in vertical and not in horizontal position. It was surmised that in vertical position the motile embryo would occur in the upper part of the egg and as a result be impaired by the injection needle. But it may plead against this that in the eggs which have lain some days in the incubator the lower surface of the air space is not even but bends downward convex. Thus the embryo will not occur medially under the air space but more obliquely; this can be observed during transillumination. In order to see more clearly into this two thousand eggs have been inoculated in vertical position against a same number in horizontal position, other conditions being equal. In this experiment no difference between both groups could be ascertained; in both the mortality was on a same level.

It ultimately appeared that in this lethal phenomenon another

factor played a major part, *viz.*, a toxine which will be discussed more in detail along with the egg to egg inoculation with Rickettsiae.

The fertile eggs after having been inoculated with Rickettsiae material are placed in an incubator at 37° C. Initially these eggs as well were viewed and turned over dayly, such as it has been described for the uninoculated eggs at 39° C. More recently this has been left of and the infected eggs are left completely undisturbed. The impression is gathered that in this way more eggs reach the final term of the period favourable for the cultivation of Rickettsiae.

When the eggs after this period are apparently still in living condition, they will be opened. This is practised by means of an electric burning apparatus, designed by SCHÜFFNER (17), a kind of thermocauter, by means of which the egg shell is burnt open along the limits of the air space. This method is assuredly to be preferred above the cutting open of the eggs. First of all sterility is guaranteed better, but moreover the experimentator is no longer in danger of infection. In fact along with the cutting minute particles of the infected shell may easily break of which may spread in all directions. GILDEMEISTER and HAAGEN (9), state in our opinion rightly that the danger for the investigator actually occurs during the experiments with the eggs. Our experiences point in a same direction; only after the introduction of the eggs in the proceedings infection occurred.

The egg once opened its content is poured out in a sterile petri dish. Of the content of the egg only the yolk sac is used for the production of vaccine. It has been ascertained that other parts of the egg contain merely few Rickettsiae.

Two smears are made out of the yolk sac. They are stained and examined microscopically as to the amount of Rickettsiae and conclusions drawn as to the density of Rickettsiae in the yolk sac. Before staining the smears are dried, either during 24 hours at room temperature or about 30 minutes slightly heated. One smear is stained according to CASTANEDA, the other according to GIEMSA (as modified according to GRACIA MIGUEL).

The CASTANEDA staining consists in a staining with a buffered methylene blue solution (pH 7) and a counterstaining with safranine (2). After many failures, indicating that the pH 7 as mentioned for the stain is of chief significance, now hardly any more failures occur. Still the staining with the GIEMSA solution is always applied as well, as in the first place it is useful as a complimentary test and in the second place it is very favourable for the judging of the preparations.

The staining according to CASTANEDA, however, is much finer than the GIEMSA staining, as the former results in Rickettsiae stained a deep blue which contrast sharply with the pink underground, whilst in the latter case they contrast more slightly with their surroundings. Often in this case the Rickettsiae have to be recognized by their form, which requires a much larger practice. In

judging the values ascertained in the preparations, the classification as it has been put up by GILDEMEISTER and co-workers (9, 10) has been adopted, which comes down to the following:
± = one single Rickettsia in the microscopic field
+ = 1 — 5 Rickettsiae per microscopic field (magnification 900 ×)
+ + = 5 — 10 „ „ „ „ „ „
+ + + = more than 10 Rickettsiae per microscopic field (magnification 900 ×)

Merely yolk sac smears which have been marked as + + or + + + may be used for preparation of vaccine. Needless to say that this method is hardly satisfactory, but as long as it has not been substituted by a better one, it will have to remain in use.

The yolk sacs are suspended in saline and these suspensions are ready for further egg inoculation, treated more in detail furtheron, whilst for the preparation of vaccine formaldehyde or phenol is added.

It is rare that at the first transmitting of Rickettsiae from the guinea pig into egg a sufficient density of Rickettsiae for the preparation of vaccine is attained at. Very often not a single one or at most a number of Rickettsiae marked merely as „+" will be revealed. When however further transmittings into egg are made, it may be noted that even in those cases where in the original material not a single Rickettsia had been detected, after repeated transmittings very rich cultures may develop. Along with this more profuse development of Rickettsiae the rate of growth will be seen to increase, so that it will no longer be needed to wait 10 days before opening the eggs; 5 days will suffice.

It might be expected that for further egg inoculations it would be needed to start from suspensions of yolk sac rich in Rickettsiae, *viz.*, from those marked „+ +" or „+ + +". This, however, is by no means the case. It has in fact been ascertained by GILDEMEISTER and HAAGEN (9) and as well by OTTO and BICKHARDT (13) that in material overgrown richly with Rickettsiae a toxine occurs which may kill of rapidly the newly inoculated eggs. The real nature of this toxine is up till now undefined. It appears noteworthy that this substance cannot be separated from the Rickettsiae by centrigugating, a fact which even renders the term „toxine" dubious.

It is obvious that this toxine was a source of difficulties in our initial experiments as we for transmitting actually made use of suspensions of yolk sacs heavily infected with Rickettsiae. Once acquainted with the cause of the difficulty it could be prevented in many cases. Its complete avoidance, however, is not possible, as it may occur sometimes that none but yields marked with + + + are available.

In order to keep down the loss as low as possible, two different roads were followed, *viz.*, either the liquid was diluted as far as possible, or the suspension was kept in the refrigerator at 4° C.

In the dilution method the number of Rickettsiae per unit of volume is diminished, which causes no difficulty as a suspension

marked „+" is already suitable for transmitting. But, and this is of more importance, the concentration of toxine is diluted, which means a lesser influence on the inoculated egg. In the refrigerator method as a matter of fact the same end is reached, the number of Rickettsiae per unit of volume decreasing along with a decrease in the concentration of toxine. In fact at low temperature, this very labile toxic substance is converted, at all events it disappears, so that without risking an early death of the embryo the eggs may be inoculated therewith. Either method has its advantages and disadvantages: it may happen that in the dilution method the influence of the toxine is not sufficiently weakened and in the refrigerator method that along with the toxine too many Rickettsiae are destroyed.

As has been stated the vaccine is prepared out of the „+ +" and „+ + +" yolk sac suspensions. In that end enough 5 % phenol is added to the suspensions to make up a 1 % phenol salt solution. Subsequently they are kept during eight days in a refrigerator at 4°C. in order to destroy any toxine eventually present.

Twelve yolk sac suspensions are mixed together. This is followed by a purification of the vaccine. These suspensions contain naturally many products valueless for the vaccine, which have to be removed as far as possible. This is done by centrifugating in the following way. Initially solid substances, so the Rickettsiae as well, are precipitated by means of centrifugating one hour on 8000 r.p.m. The supernatant valueless liquid is removed. The sedimentation is brought once again in suspension and centrifugated 10 minutes on 3000 r.p.m. By these means the coarser particles out of the sediment are precipitated. The supernatant liquid (now the sediment is discarded) in which the major part of the Rickettsiae occurs, is centrifugated over again one hour at 8000 r.p.m. The sediment thus obtained is subsequently brought in suspension in saline containing 0.5 % phenol in the ratio of 25 ml per yolk sac. The resulting product is the typhus fever vaccine, such as it is applied nowadays.

Obviously it will, however, have to pass several controls before it may be issued. Among these we mention: the control on sterility, the control on harmlessness, and finally the control on its immunizing value. As far as sterility is concerned, as a matter of fact all different stages of the vaccine are under control. First of all the sterility of the yolk sac suspension before phenol has been added, is ascertained in the usual way aerobically and anaerobically. Merely those yolk sac suspensions will be further worked with in which no contamination either by means of stained smears or by culturing could be ascertained. After phenol has been added the suspensions are kept during eight days in the refrigerator, followed by a control of sterility. No irregularities having been noted, after the twelve suspensions have been mixed together, again the sterility is ascertained. After centrifugation the sterility of the purified vaccine is tested once more. If sterile, the vaccine is distributed over ampullae and by means of a test at random the sterility is finally established.

The control of harmlessness consists in the intraperitoneal injection of the vaccine in 3 guinea pigs. These 3 animals may not during an interval of 3 weeks show any raise of temperature, which might remind of typhus fever. Initially we had gladly increased the number of animals, but under present conditions this could not be realised. The experience of recent years has taught, however, that 3 may suffice.

Simultaneously with the control of harmlessness the immunizing value is determined. In this end 5 guinea pigs are immunized with the vaccine. A month later these guinea pigs along with 5 normal animals (which as far as possible originate from the same batch as the immuzined) are injected intraperitoneally with Rickettsiae containing material. Moreover as far as possible unto these 10 guinea pigs 5 are added which had already been infected with typhus fever in an earlier experiment and had then shown a temperature curve specific for typhus fever. The results of the experiments on immunity, obtained at up till this moment, are presented in the following table.

| | Experimental animals | Total number of guinea pigs | Typhus fever curve | Delayed typhus fever curve | No raise in temperature | Not got hold of infections in % |
|---|---|---|---|---|---|---|
| 1. | Guinea pigs immunized with typhus fever | 77 | 2 | 5 | 70 | 91 |
| 2. | Control guinea pigs | 60 | 40 (4 dead) | 13 | 7 | 11 |
| 3. | Reinfected guinea pigs | 40 | 1*) | 2**) | 37 | 92 |
| 4. | Guinea pigs for transmitting of strain | 739 | 517 | 91 | 70 | 9 |

*) Reinfection 5 weeks after the end of the first febrile period.
**) Reinfection 7 to 8 weeks after the end of the first febrile period.

Under 4 is inserted in this table a survey of the course of the infection in normal guinea pigs as it has been observed for the usual transmittings of the strains during the two later years.

It may be noted that in merely 9 % of normal guinea pigs the infection does not get hold. The percentage of 11 as inserted under 2 is thus on the high side; it may have been caused either by too small a number of animals in this group, or by a difference in susceptibility for the infection in the various batches of guinea pigs. Anyhow the 91 % out of the vaccinised guinea pigs in which the infection did not get hold shows clearly the immunizing action of

the vaccine. The more so as under 1 the results arrived at with the initially prepared vaccine are taken up as well. In the latter vaccines the demands on the control of value (number of Rickettsiae per microscopic field) had been less severe than in later experiments which will tell in the lesser immunizing effect. It thus may certainly be assumed that the 91 % as noted for the resistence against the infection is by no means flattered.

Moreover I wish to point to the fact, that in 5 of the immunized guinea pigs a delayed febrile curve occurred. In the non-treated animals this could merely be observed in 13 out of 53. Thus in immunized animals this delayed febrile curve is of more frequent occurrence.

Experience has taught that Rickettsiae which have been cultivated under unfavourable condition (too high or too low temperature) when injected into guinea pigs, may often induce a delayed febrile curve. The occurrence of such a temperature curve in vaccinised animals indicates in our opinion the fact that an inhibiting action is excercised on the Rickettsiae, which may lead to the conclusion of an existing immunity, although unsufficient, in these animals.

Was, however, a result of 100 % to be expected for the vaccination? To answer this question it has been inserted in the table under 3, what happened when guinea pigs which had already gone through a typhus fever were inoculated over new. In 3 out of 40 reinfected guinea pigs again a febrile curve arose; even the going through a typhus fever does not induce in guinea pigs 100 % immunity. Thus the answer to the above question has to be in the negative.

As far as the results of vaccination in man are concerned, we cannot give evidence based on experience of our own. We thus have to limit ourselves to literature.

In a publication of Ding (5) a summarizing discussion is to be found which presents a good survey of the results attained at up till now (see also Eyer (8)).

It appears that although vaccination against typhus fever does not decrease the number of cases, the disease takes in most of the cases a much less serious course than without vaccination. Finally special attention is drawn to the fact that no fatal cases occur among the vaccinised patients. The course of disease in two cases of typhus fever among our laboratory workers corresponds with this sentence.

Next to the preparation of egg vaccine the preparation of so called lung vaccine was taken on hand (7). The cultivation of Rickettsiae on mice lung did not offer technical difficulties. The growth was very profuse, often at least as profuse as in egg. After the lung had been ground down and phenol had been added a sterile vaccine could be obtained. It was, however, unsatisfactory that the sterility tests of the suspension of lung substance always resulted in the ascertainment of a contamination. It is true that it could be eliminated by means of phenol or eventually formaldehyde, but it seems

to us undesirable to make use of such a vaccine. As in the preparation of vaccine such as it has been described above, such contaminations are ruled out, the latter method is preferred above the preparation of the lung vaccine. Moreover it is much easier to dispose of a sufficient number of eggs than of mice. We mean to be all the more entitled to this conclusion in view of the following sentence in a publication of BIRAUD (1): „En effet, la méthode de culture sur embryons de poulets et celle sur poumons de rongeur sont également capables de fournir du vaccin en quantité et à l'heure actuelle, on ne peut pas prouver que le vaccin préparé par une de ces méthodes soit plus efficace que celui obtenue par l'autre".

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(From the „Rijks Instituut voor de Volksgezondheid" at Utrecht).

# THE ANTIGENIC PROPERTIES OF BACTERIAL SPORES

by

**J. H. BEKKER**

(Received February 14, 1944).

A great number of researches have made it evident that a bacillus may be the bearer of various kinds of antigens, which, according to their properties, are designed as H-, O-, Vi-, L-antigens etc. Only a few authors have taken up the question whether the spores of the bacilli possess antigenic properties and ascertained whether an injection of spores into the blood of an animal produces antibodies specific for the spores and not for the bacillary forms.

The results of these experiments are contradictory. DEFALLE (2) working with *B.mycoides*, *B.mesentericus*, *B.subtilis*, *B.alvei* and two strains of low virulence of *B. anthracis*, MELLON and ANDERSON (5) working with *B. subtilis* and HOWIE and CRUICKSHANK (3) with *Cl. sporogenes*, *B. mesentericus* and two strains resembling *B. cereus*, are of the opinion that spores and bacilli each produce their specific antibodies, whereas STARIN and DACK (7) working with *Cl. botulinum*, *Cl. sporogenes* and *Cl. putrificum*, as well as KRAUSKOPF and MCCOY (4) working with *B. niger* state that spores only produce antibodies to the bacillary forms.

I thought it interesting to investigate in a similar way a few different representatives of the genus *Bacillus*, *viz.*, an avirulent strain of *B. anthracis* (Bloed 1916), *B. ubiquitarius* and *B. mesentericus*.

TECHNIQUE.

In order to obtain asporogenic bacilli the strains were incubated on bouillon agar at 42° C. as previously described (1). Asporogenic strains were obtained of *B. anthracis* (Bloed 1916) and *B. ubiquitarius*, and the forming of spores could, if not entirely, still in a very considerable measure, be suppressed in *B. mesentericus*. H- and O-antisera were induced in the usual way in rabbits by means of *B. ubiquitarius* and *B. mesentericus*, whereas for *B. anthracis* (Bloed 1916) only an antiserum to the living bacilli was made.

Almost complete sporulation was obtained with *B. ubiquitarius* and *B. mesentericus* by incubating the cultures for 20 days at 37° C.

on asparaginate agar as described by Howie and Cruickshank. As to *B. anthracis* (Bloed 1916) we detected in the films always some, though degenerated bacillary forms. Spore-antisera were prepared by 6 intravenous injections of 1 cc of a suspension of the growth of 5 asparaginate agars in 15 cc saline on successive days in a rabbit followed by 4 injections on successive days after an interval of a week; the animals were bled 4 days after the last injection. In a similar way spore-antisera were made by means of spores killed by autoclaving at 120° C. for half an hour.

Stable suspensions for the agglutination tests were obtained by a somewhat simplified technique of Noble (6) *viz.*, by shaking the bacterial suspensions in a mechanical shaker for 24 hours and allowing it to stand for 6 hours in which lapse of time the larger clumps will precipitate; the homogeneous supernatant liquid was used as suspension for the agglutination tests. For the spore suspensions these periods amount to 3 resp. 2 hours. The agglutination tests were incubated at 37° C. for 20 hours.

Results.

The results of the experiments are given in the following tables:

Table 1

Strain: *Bacillus anthracis* (Bloed 1916)

| Antiserum / Suspension | living bacilli | living spores |
|---|---|---|
| living bacilli | 1 : 640 | 1 : 40 |
| living spores | nil | 1 : 320 |

Table 2

Strain: *Bacillus ubiquitarius*

| Antiserum / Suspension | H-bacilli | O-bacilli | living spores | autoclaved spores |
|---|---|---|---|---|
| H-bacilli | 1 : 640 | nil | 1 : 20 | 1 : 20 |
| O-bacilli | 1 : 20 | 1 : 320 | nil | nil |
| living spores | 1 : 20 | nil | 1 : 1280 | 1 : 640 |
| autoclaved spores | nil | nil | 1 : 320 | 1 : 320 |

Table 3

Strain: *Bacillus mesentericus*

| Antiserum / Suspension | H-bacilli | O-bacilli | living spores | | autoclaved spores |
|---|---|---|---|---|---|
| | | | un-absorbed | absorbec with living bacilli | |
| H-bacilli | 1 : 2560 | nil | 1 : 1280 | nil | 1 : 40 |
| O-bacilli | nil | 1 : 2560 | 1 : 40 | | 1 : 80 |
| living spores | 1 : 80 | 1 : 80 | 1 : 1280 | 1 : 1280 | 1 : 640 |
| autoclaved spores | nil | nil | 1 : 1280 | | 1 : 640 |

Table 4

| Antiserum / Suspension | living spores of *B.anthracis* (Bloed 1916) | living spores of *B.ubiquitarius* | living spores of *B.mesentericus* |
|---|---|---|---|
| living spores of *B.anthracis* (Bloed 1916) | 1 : 320 | 1 : 20 | 1 : 20 |
| living spores of *B.ubiquitarius* | 1 : 20 | 1 : 640 | 1 : 40 |
| living spores of *B.mesentericus* | nil | 1 : 20 | 1 : 1280 |

Discussion.

From the tables 1, 2 and 3 it appears that the spores of the three strains tested possess antigenic properties. The spore antisera show besides the antibodies to the spores by means of which they have been prepared, no or only very few antibodies to the bacillary forms to which these spores give rise on germination. With *B. mesentericus* (Table 3) the living spore antiserum contained also many H-antibodies, possibly as a consequence of the small number of living bacilli occurring in the spore suspension or perhaps owing to the fact that some spores had germinated into bacilli in the body of the animal already. After absorption of this spore antiserum with living bacilli these antibodies had disappeared, whereas the spores were still agglutinated up to the titer.

Further it appeared that the bacillary antisera contained no or very few antibodies to the corresponding spores.

These facts may be considered as a proof for the existence of a specific spore-antigen that can clearly be distinguished from the antigens of the bacillary forms.

From the tables we can also see that autoclaving during half an

hour at 120° C. has little or no effect on the antigenic properties of the spores.

Finally I investigated whether the antigens of the tested spores differed mutually or whether there existed only one special spore-antigen. From table 4 it appears that the spores of the strain of *B. anthracis* (Bloed 1916), *B. ubiquitarius* and *B. mesentericus* produced antibodies distinct from each other, so that the spore-antigen of these strains are also distinct.

## Summary.

1°. Bacterial spores are antigenic.
2°. Spore-antigen is distinct and separate from the antigens of the bacillary forms to which the spores give rise on germination.
3°. Antigens of the spores of various kinds of spore-bearing bacilli are also mutually distinct.

I am greatly indebted to Miss A. de Groot for her technical assistence.

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(Du laboratoire d'hygiène, Université de Groningue).

# MÉTHODE SIMPLE POUR LA DESSICCATION DANS LE VIDE DE CULTURES BACTÉRIENNES

par

**A. E. BEUTE**

(Reçu le 17 Février 1944).

La dessiccation dans le vide et à température basse de produits biologiques, dans l' intention de les conserver, est en usage depuis longtemps et avec succès. Un résumé détaillé de la littérature a été donné e.a. par FLOSDORF et MUDD (3, 4).

Un appareil simple, construit principalement pour la dessiccation de cultures bactériennes, peut avoir une grande importance pour beaucoup de laboratoires, comme économie de temps et de milieux de culture. D'ailleurs les cultures séchées ne changent pas de nature; il n' y a ni dégénération, ni perte de virulence, conséquences bien connues et redoutées des repiquages répétés.

Avec le procédé en question ce sont surtout les détails pratiques qui sont intéressants. Voici une description brève de la méthode facile et de l'appareil simple, en usage depuis deux ans, pour la dessiccation de cultures bactériennes au laboratoire d'hygiène à Groningue.

L' appareil est basé sur le modèle de COOPER et GRABILL (2); cependant la construction et la méthode d'emploi sont assez modifiées. Ainsi le temps nécessaire pour la dessiccation a été ramené d'environ 24 heures jusqu' à une heure, l'appareil est plus robuste et surtout, en remplaçant la dessiccation d'une quantité de liquide par le dessiccation de bandelettes de carton buvard, trempées de liquide, la maniabilité est augmentée et les risques de contamination sont diminuées.

Appareil.

L'appareil à sécher se compose de deux parties principales:

1. La pompe à vide, munie d' un flacon de desséchage et des manomètres.

2. L'appareil à sécher proprement dit.

1. La pompe (a), une pompe à huile Cenco Hyvac, avec un flacon de desséchage (c) pour protéger l'huile contre les dernières traces d'humidité, et le moteur (b) sont montés sur une planche, ainsi qu'un manomètre fermé à mercure (d), un petit manomètre Mc Leod (e) et la conduite, servant à faire le vide, en cuivre (f). Presque

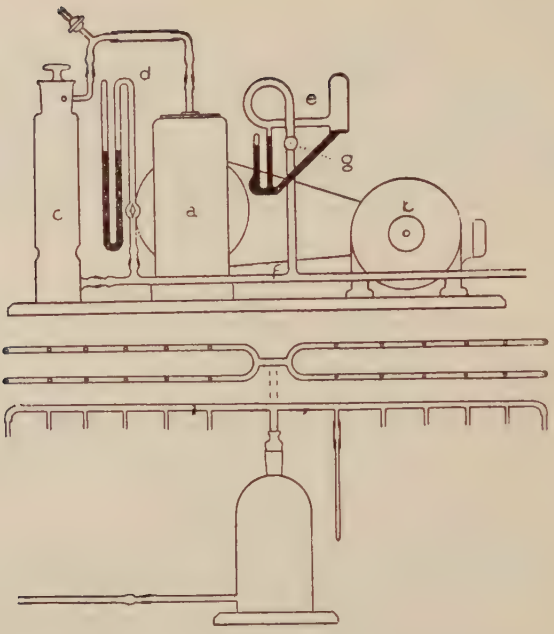


Fig. 1. Schéma au douzième environ. En haut la pompe à vide avec accessoires, en bas l'appareil à sécher propement dit.

toutes les jonctions sont des jonctions à l'émeri normalisées 11/10. Il est nécessaire d'employer pour le graissage des jonctions une graisse spéciale pour le vide; nous employons celle d'après Ramsay. Le bouchon à l'émeri du flacon de desséchage est perforé et sert de robinet entre la pompe et la conduite servant à faire le vide. Le tube de connexion entre la pompe et le flacon de desséchage porte un tube latéral avec robinet, pour donner accès à l'air. Le manomètre Mc Leod est un „drehbares Hochvacuummanometer nach Dr. Brunner" (1); fabrication de Bender et Hobein, München. Pour faire un mesurage, on tourne l'instrument maniable sur sa jonction à l'émeri (g). Les pressions peuvent être mesurées jusqu'à 0.001 mm.

2. L'appareil à sécher est en cuivre et se compose d'un vaisseau cylindrique et des bras porte-tubes. Le vaisseau a une hauteur de 18½ cm (jusqu'au col) et un diamètre de 12 cm. Dans la paroi, un peu au dessus du fond, un tube avec une jonction noyau à l' émeri 11/10, en cuivre jaune, est soudé, pour faire connexion avec la conduite à faire le vide. Le col du flacon est une jonction manchon à l'émeri 25/10 en cuivre jaune. Là s'emboitent les bras porte-tubes, avec des jonctions noyaux à l'émeri 25/10 en cuivre jaune. Les bras sont des tubes horizontaux, portant de petits tubes verticaux, munis de petites pièces de caoutchouc à paroi épaisse. Les

tubes de verre à évacuer peuvent être poussés dans ces dernières. Nous employons un porte-tubes avec un bras horizontal, muni de six tubes verticaux et un porte-tubes avec quatre bras horizontaux, munis au total de 24 tubes verticaux. Chaque tube horizontal est pourvu d'une petite tablette noire, pour écrire avec de la craie. En dehors de son emploi, le flacon est fermé d'un bouchon en cuivre jaune avec jonction à l'émeri 25/10. Le vaisseau en cuivre, comme le flacon de desséchage devant la pompe, sont remplis de drierite, du sulfate de calcium anhydre, fixant très vite l'eau, jusqu'au demi-hydrate. La tension de la vapeur de cet hydrate à 25° C. n'est que 0.004 mm. Drierite garde sa porosité en fixant l'eau. La régénération est très facile: en chauffant de 160 à 200° C.[1]).

Au lieu de drierite, on peut, probablement, se servir de silica-gel, mais nous ne l'avons pas expérimenté.

Méthode.

Chaque souche bactérienne est séchée au sextuple, pour avoir quelques réserves en faisant des cultures nouvelles.

L'emploi des milieux de culture solides est le plus simple. La culture d'un tube à milieu incliné, ou, en cas de cultures minces, de deux ou trois de ces tubes, est émulsionnée dans $\frac{1}{2}$ à $\frac{3}{4}$ cm$^3$ de bouillon, de manière qu'une émulsion épaisse en résulte. Si la culture en milieu liquide est indispensable, il faut centrifuger les microbes et les émulsionner de nouveau dans $\frac{1}{2}$ cm$^3$ du liquide surnageant. Ceci est facilité si le tube de centrifugation est pourvu par avance de trois perles de verre. Dans l'émulsion épaisse, dont la pureté est controlée au microscope et qui est transportée dans un petit tube stérile, sont trempées six petites bandes de carton buvard, d'une largeur de 25 × 3 mm et d'une épaisseur de 0.9 mm. Ce carton buvard doit être pur et neutre; nous employons la marque „Vampier". Chacune des bandelettes, bien trempée de l'émulsion bactérienne, est transportée, avec une pincette dont les mords sont flambés, dans un tube de verre stérile, long de 16 cm, avec fond rond, tiré des tubes pour pipettes Pasteur (diamètre externe 0.6 à 0.8 cm). A l'aide d'une anse longue on pousse la bandelette jusqu'au fond. Le coton des tubes est coupé, de sorte qu' il en reste un demi cm; cette partie est rentrée un peu dans le tube.

On fait glisser les tubes, munis de bandelettes, à l'aide d'un peu de glycérine dans les pièces de caoutchouc de l'appareil à sécher.

Avec le porte-tubes aux quatre bras quatre souches peuvent être séchées à la fois. Un bloc de bois, pourvu de 4 rangées à 6 cavités, permet de manier facilement les 24 tubes, sans risquer de confusion. Sur ce bloc il y a une marge noircie, pour écrire avec de la craie.

Ensuite on fait le vide, la pression doit baisser au dessous de 0.2 à 0.3 mm de mercure; une bonne pompe atteint aisément quelques centièmes de mm. Une heure de desséchage suffit large-

[1]) Le produit est mis en circulation par W. A. Hammond, Yellow Springs, Ohio (U.S.A.). Représentant aux Pays Bas: Handelsonderneming v/h J. & W. Wegman, Staalkade 1, Amsterdam.

ment. Pendant ce temps on fait fonctionner la pompe. Au commencement on peut bien éprouver, à l'extérieur des tubes, le refroidissement des bandelettes et assez souvent le voir à une buée.

Après une heure on sépare par la fonte, en maintenant le vide, les 6 à 8 cm du fond des tubes, dans lesquels se trouvent les bandelettes. Pour cela est employé un petit brûleur avec deux flammes fines, croisantes, fait d'après les directions de FLOSDORF et MUDD(3). Pour le verre employé, de fonte facile, l'usage de l'air comprimé est superflu, même indésirable. La séparation exige un peu d'adresse. En commençant on fera bien de rétrécir un peu, à la flamme, les tubes à l'endroit de la fonte, avant de les fixer à l'appareil à sécher; après quelque exercise ce n'est plus nécessaire. Le verre ne doit pas coller des deux côtés, dans ce cas les tubes crèvent toujours après quelque temps; il faut chauffer uniformément de tous côtés, en tirant au fond du tube. Le bout est arrondi spécialement à la flamme.

Dans les tubes ayant une fuite, les microbes étaient toujours morts. On peut contrôler le vacuum des tubes avec un inducteur approprié; dans le vide il se produit une lueur violette.

Sur les tubes sont écrites, d'un pinceau fin et d'une laque à prise rapide, la signature de la souche et la date du séchage; les six tubes d'une souche sont réservés dans une boîte en fer blanc, comme on s'en sert pour expédier des matières infectées. Un petit billet ci inclus peut contenir des renseignements plus détaillés. Ainsi une souche, séchée au sextuple, ne demande pas plus de place qu'un tube de culture ordinaire.

Le vaisseau de cuivre contient 2 kg de drierite, pouvant fixer 132 g d'eau (6.6 %). Il vaut mieux n'utiliser que la moitié de cette capacité. Six bandelettes de carton buvard absorbent environ 0.4 g d'eau, de sorte qu'on peut sécher 165 souches bactériennes avant que la régénération du drierite devienne nécessaire. Cela se fait en chauffant le vaisseau ouvert pendant 12 heures de 160 à 180° C.

Une personne peut facilement sécher 12 souches par jour, chacune comprenant six tubes.

Nous avons réservé les souches séchées pour la plupart à la glacière; une partie plus petite est restée à la température ambiante, mais dans l'obscurité. Jusqu'à présent nous n'avons pas observé de résultats différents.

Si l'on désire une culture nouvelle d'une souche séchée, on flambe le bout d'un tube et on le casse avec une pince dont les mords sont flambés; puis on coupe le tube un demi cm au dessus de la bandelette. Avec une pincette aux mords stériles, la bandelette est transportée dans un milieu liquide ou dans l'eau de condensation d'un milieu solide. Après l'incubation nécessaire la culture s'est développée de nouveau: en cas d'un milieu solide on peut étendre les colonies qui se trouvent autour de la bandelette sur toute la surface du culot.

Nous avons séché de la manière décrite plus de 170 souches de *Haemophilus meningitidis* et *Haemophilus influenzae*, ainsi que

plusieurs souches des genres *Neisseria, Diplococcus, Streptococcus, Lactobacillus, Vibrio, Salmonella, Eberthella, Shigella, Listerella, Bacillus, Corynebacterium* et *Fusobacterium*, aussi des levures. Nous n'avons eu qu'un échec, à savoir chez les Leptospires. Du reste une nouvelle culture a toujours réussi à partir d'un tube bien scellé. Le résumé suivant montre les résultats obtenus en ce qui concerne la durée de la vie des souches séchées, jusqu'a présent:

| Espèce bactérienne | Séché pendant | Conservé à |
|---|---|---|
| *Haemophilus meningitidis* et *Haemophilus influenzae* | 17 à 24 mois | glacière et temp. ordinaire |
| *Neisseria gonorrhoeae* | 16 ,, | glacière |
| *Neisseria intracellularis* | 12 et 17 ,, | ,, |
| *Diplococcus pneumoniae* | 17 ,, | ,, |
| *Corynebacterium diphtheriae* | 15 ,, | ,, |
| *Vibrio comma* | 12 ,, | temp. ordinaire |
| *Salmonella schotmuelleri* | 16 ,, | glacière |
| *Shigella alcalescens* | 12 ,, | temp. ordinaire |
| *B. megatherium* mutilat de DEN DOOREN DE JONG | 12 ,, | glacière |

## Sommaire.

Dessiccation dans le vide de cultures bactériennes, par procédé simple et rapide.

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# ABSTRACTS

A. Quispel, The mutual relations between algae and fungi in lichens. Diss. Groningen, 1943. Recueil des Trav. bot. Néerl. **40**, 413, 1943.

The lichen-symbiosis was investigated by means of experiments with pure cultures of the components. As lichen-algae some *Cystococcus* species were isolated, the only lichen-fungus investigated was *Xanthoriomyces parietinae*. As an orientation, however, a great many experiments were performed with the fungi which are living in symbiosis with the aerial algae *Pleurococcus* and *Apatococcus*, as it appeared that these fungi are closely related to true lichen-fungi, whilst their growth-velocity is much better. In consequence they are an excellent object for the study of the lichen-symbiosis. As far as possible the results obtained with the investigation of these fungi were tested upon *Xanthoriomyces*.

It appeared that the fungi did not develop in synthetic culture solutions without the addition of certain nutrilites (aneurin, $\beta$-alanin and other bios substances). The lichen-algae can provide the fungi with these nutrilites. These algae themselves were stimulated by the addition of asparagin, nicotinic acid and certain bios substances, when developing in organic culture solutions. In inorganic solutions a good development could only be obtained after the addition of a small amount of ascorbic acid. It is very probable that the lichen-fungi are able to stimulate the photosynthesis of the algae by the production of ascorbic acid or a related substance.

The fungi did not produce lichenic acids in cultures. On the other hand the alga *Apatococcus minor* synthesizes a remarkable metabolic product, called apatococcin, which most probably is related with certain aliphatic lichenic acids.

An investigation of the water-household of some lichens showed that the protective influence of the fungus against a desiccation of the algae is merely very small and can only be perceived when the desiccation is not too intense.

The final conclusion is that the lichen-symbiosis may be regarded as a „mutualistic symbiosis" in which the exchange of nutrilites plays an important role. A. Q.

(From the Laboratories of the City Health Department, Amsterdam)

# OBSERVATIONS ON 'HEALTHY' HUMAN CARRIERS OF *PLASMODIUM VIVAX*

by

**F. J. A. PAESI**

(Received December 30, 1943)

With the term 'healthy' parasite carriers persons are designed harbouring parasites, which, however, do not cause them sufficient trouble to make them seek medical advice. So such persons are not actually healthy, although they are supposed to be so.

The extensive investigations of SWELLENGREBEL c.s. have made it clear that these 'healthy' carriers are of paramount significance for the epidemiology of benign tertian in this country. VAN THIEL (6) and SWELLENGREBEL and DE BUCK (4) have demonstrated i.a. that practically one subspecies of *Anopheles maculipennis* only acts as an intermediate host of *Plasmodium vivax* in our country and that the activity of the mosquito with respect to transmission is mainly confined to the second half of August and to September and October. In this period, however, the number of malaria patients (*viz.*, those, suffering from clinically recognisable accesses of fever) is small; these are met with mainly from May up till August.

So in most cases the mosquitoes will ingest the germs with which they may subsequently infect healthy persons not from diseased persons, but from 'healthy' carriers, who have suffered from malaria at an earlier date. Persons, who are infected in autumn in this way, will generally not incur their initial attack before the summer of the following year, after a period of incubation of about 9 months.

Thus another group of 'healthy' parasite carriers is formed, *viz.*, those who may expect an outbreak of the disease. This group is probably of no significance for the spread of malaria (see further on).

The important part played by the parasite carriers in the spread of benign tertian induced us in the summer 1940 to take up an investigation of the number and nature of the parasites in their blood and to collect some data on their state of health. This investigation, which has been carried out in the northern part of Amsterdam, furnished, moreover, the opportunity to judge the results of the 'autumnal quininisation'. This treatment, which was applied to all those who had suffered from one or more attacks of

fever in summer, consisted in the administration of a dose of 1 g of quinine (dose for adults) once a week, with the aim of inducing a decrease of the number of parasites in the circulating blood to such an extent that the chance of infection for mosquitoes in autumn would be markedly diminished. By such means it was hoped to stop the spreading of the prevailing epidemic, as far as Amsterdam was concerned.

For our investigation we chose a suburb in the extreme north of Amsterdam, where a great number of cases had occurred in 1939 and the beginning of 1940. This suburb was mainly populated by unemployed; these were treated by Dr. BERGHUIS, whom we are greatly indebted for his advices. In about 1200 visits thick films were made, which were then stained in the usual way by Giemsa's solution. Subsequently the number of parasites per 6000 leucocytes was counted — as is usual here — and their stage of development noted.

## I CARRIERS DURING THE PERIOD OF INCUBATION

In June and July 1940 we have been looking for carriers of parasites, who might have been infected in the autumn 1939 and whose first attack was still to be expected, in families in which the number of malaria patients had been particularly high in the preceding year. We found among 174 members of 21 families 39 carriers of parasites, among whom, however, three only had not yet suffered from an attack of fever. (The others were persons, who had had their first attack already in 1937, 1938, 1939 or — as the investigation was started somewhat late in the season for malaria — shortly before, in 1940). Of those three some data are presented:

Table I

| | A | B | C |
|---|---|---|---|
| Number of days which elapsed before the attack | 6 | 5 | ½ |
| Number of parasites per 6000 leucocytes | 240 | 1 | 4500 |
| Nature of the parasites | young rings<br>fullgrown rings | young rings | fullgrown rings<br>young rings<br>♂ gametocytes |

C had his first attack some hours after his blood had been tested. For various reasons it was impossible to examine A's and B's blood at regular intervals up till the first attack. Among the 174 persons examined occurred, next to these three, a further e i g h t in whom n o parasites could be detected, although their first attack occurred after a lapse of 3 days(!), 2, 2, 2, 3, 4, 4 and 4 weeks respectively.

These facts seem to indicate that during the period of incubation up till a short time before the attack no parasites, or only a very small number, occur as a rule in the circulating blood. This is in agreement with the experience previously acquired in voluntarily infected persons as well as in sufferers from genuine paralysis, who had been inoculated with germs of benign tertian (SCHÜFFNER, KORTEWEG and SWELLENGREBEL (3)). It is well known that even during KORTEWEG's initial remittent no or few parasites can be traced in the blood (2).

## II PARASITE CARRIERS WHO HAVE ALREADY SUFFERED FROM ACCESSES

### The autumnal quininisation

Quinine was administered from August 15th till November 1st 1940. The weight of the dose had been fixed according to results arrived at by KORTEWEG (2) in Noord-Holland and by KOCH c.s. (1) in Africa and Italy. Their communications, however, bore on the administration of 1 g of quinine (dose for adults) on at least 2 succeeding days, *viz.*, every 9th and 10th, or every 6th and 7th day, the treatment being concerned with the prevention of relapses or of new infections. As the autumnal quininisation aimed at keeping parasites out of the circulating blood only, 1 g of quinine once a week was considered worth trying. More recent literature did not furnish any further encouragement.

In July and August 54 carriers of parasites were traced, who, from August 15th onward, were supposed to take the drug weekly. Among these carriers, fifteen, the blood test of whom was repeatedly positive in October, were selected for judging the effect of this particular mode of administering quinine.

As this effect was still unknown to us, we had told all of them temporarily to stop taking the weekly doses of medicine on October 1st in order to be able to trace a number of 'good' carriers of parasites. In the fifteen who were found suitable in the weeks which followed, the weekly treatment was resumed from November 1st onward (the actual quininisation was then already brought to a close).

Twelve of these fifteen had had their first attack in 1940, two of them in 1938 and one in 1939. From November 4th up till November 30st blood films were made daily, except on Sundays; on Sundays, however, they had to take the quinine.

We did not enforce the taking of the drug. An eventual success of such a compulsatory administration would not have taught us anything about the effectiveness of the method, as the actual taking of the medicine will always have to be left to the initiative of those concerned.

Nevertheless all were reminded on Saturday of their Sunday duty and each of them was asked on Monday, whether it had been fulfilled; in the few cases in which this could not be affirmed one of the following days was destined for the taking of the drug.

For a separation into two groups, one taking the medicine and the other not, the numbers obviously are too small.

Parasite counts (number of parasites per 6000 leucocyte

| | October 1940 | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 28 | 29 | 30 | 31 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| T. S. ..... | | | | | 48 | | □ | — | — | 1 | — | — | — | □ |
| A. v. D. .... | | | | | | | □ | 12 | — | 20 | 1 | 6 | 1 | □ |
| D. de R. .. | | [9300] | | □ | | | □ | — | — | — | — | — | — | |
| W. de R. *) | | 60 | | | | | □ | 1 | 1 | — | 1 | — | 4 | |
| S. F. ..... | | 1 | | | | | □ | — | — | — | — | — | — | |
| J. T. ..... | | 240 | | | | | □ | 1 | 5 | — | 3 | — | 1 | □ |
| J. Kl. .... | | 180 | | | | | □ | — | 2 | — | — | | — | □ |
| W. v. Z. .. | | 4 | | | | | □ | | 2 | — | — | — | 6 | □ |
| Adr.v.Z. .. | | 120 | | | | | □ | | 5 | 8 | 8 | 10 | 5 | □ |
| W. Reg. .. | 120 | | □ | | | | | | | □ | 120 | 72 | 120 | |
| T. V. ..... | | | | □ | | | | | | | | | 7 | □ |
| L. V. ..... | | | | □ | | | | | | | | | 720 | □ |
| R. v. L.... | 240 | | | | | □ | | | | | | | — | □ |
| J. Sch..... | | | □ | | | | | | | | 2400 | | | □ |
| C. V. ..... | | | | □ | | | | | | | [2] | | | |

*) Spleenless carrier.
□ Day, on which quinine was prescribed. From October 29th till November 2nd
— No parasites found.

The results were disappointing. Although all of the 15 carriers assured emphatically, to have taken the quinine regularly (with or without extra remindings), not one of them was wholly free from parasites during this period (see Table II).

Thirty-six times we had thus the opportunity to count the numbers of parasites in the blood 24 hours (in some cases a few hours) before as well as after the moment at which quinine was taken. After the taking of the medicine the number of parasites appeared in 14 cases smaller, in 12 cases larger and once the same when compared with the amount before the taking, whilst in one case it was negative before as well as after. In two out of 14 cases only in which the parasite count decreased, it came down to zero; in one of the latter cases the blood test remained negative for the period of observation, in the other during not more than 24 hours.

Never could any increase in the number of degenerated plasmodia be noted after the taking of the drug; gametocytes were always present.

So it is clear, that the method such as it has been followed has not led to its aim: the expelling of most of the malaria parasites from the blood circulation. It is self-evident, that the unsuccessful result may not wholly be ascribed to an insufficient dosage of quinine; as the actual taking had not been ascertained. As, however,

of 15 healthy carriers during the autumnal quininisation.

40

| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | □ | — | — | — | — | — | — | □ | | — | — | — | | — |
| — | — | — | □ | — | — | — | | — | — | | — | — | — | — | — | — |
| — | 1 | 1 | | 20 | 30 | 60 | 60 | 360 | 720 | | 3000 | 6030 | 1500 | 150 | — | 3 |
| — | — | — | | — | — | — | — | 1 | 5 | | 120 | — | 1 | 300 | 120 | 120 |
| — | — | — | | — | — | | 2 | — | — | | 20 | □ | 1 | 5 | — | 3 |
| — | — | — | □ | — | — | — | — | — | — | □ | — | — | | — | — | — |
| 8 | 10 | 60 | □ | 10 | 10 | | 10 | 30 | 6 | □ | 5 | 10 | 10 | | 4 | 1 |
| 5 | 90 | 90 | | 30 | 540 | 84 | 180 | 120 | 120 | □ | 2 | 1 | — | — | | — |
| 2 | | 15 | □ | 3 | 4 | | 1 | 1 | 10 | □ | 15 | 10 | 5 | 15 | | 40 |
| 80 | 48 | 540 | | 720 | 180 | 210 | 5 | 1 | 1 | | — | — | — | — | 1 | |
| — | — | 5 | □ | 20 | 15 | 10 | 1 | 60 | 8 | □ | 15 | 84 | 50 | 180 | | 1 |
| 60 | 300 | 1200 | □ | 360 | 180 | 180 | 90 | 300 | 90 | □ | 120 | 480 | 240 | 25 | | 180 |
| — | — | — | □ | 1 | — | — | — | 3 | 5 | □ | 1 | — | — | — | 4 | — |
| 1 | — | — | □ | 1 | 240 | 60 | 4 | 20 | — | | 1 | — | — | 1 | — | 1 |
| 30 | 2 | 40 | | 80 | 5 | 20 | 60 | 72 | — | | — | — | — | — | — | — |

carriers taken on their own account.

it is quite out of the question that the medicine would never have been taken by any of the carriers and as nevertheless practically no positive results could be booked, the conclusion may be drawn, that in case this method might be taken up again — either with or without supervision of the taking of quinine — a higher dose is to be recommended.

## Parasite counts in 1941

From the 15 carriers the blood film of whom had already been tested systematically during the autumnal quininisation thick films were prepared in January/February, April/May and June/July 1941 along quite similar lines: every working day during $3\frac{1}{2}$ weeks at a stretch (See table III, IV and V).

It may be said here, that not one of the carriers had an actual relapse during the period of observation.

The data thus collected all confirm the already known fact, that the number of parasites in the blood of those suffering from chronic malaria varies strongly. Moreover, the variation appeared to occur with a certain regularity. In 9 out of 15 carriers we could note a regular alternation of periods in which no or hardly any parasites could be detected, with others in which their number

Table III — Parasite counts (number of parasites per 6000 leuc

| | January 1941 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| T. S. | 2 | 1 | — | — | — | 1 | | 2 | — | — | — |
| A. v. D. | — | — | — | — | — | — | | — | — | 1 | — |
| D. de R. | — | — | — | — | — | — | | 1 | 2 | 7 | 14 |
| W. de R. *) | 90 | 72 | 420 | 120 | 60 | 96 | | 300 | 420 | 120 | 150 |
| S. F. | 15 | 10 | 2 | 1 | — | — | | — | — | — | — |
| J. T. | — | — | 3 | — | 2 | 2 | | 10 | 4 | 12 | 5 |
| J. Kl. | — | 2 | 4 | 2 | — | 2 | | — | — | 2 | 10 |
| W. v. Z. | 90 | 48 | 240 | 130 | 360 | 25 | | 6 | | — | — |
| Adr. v. Z. | 4 | 3 | 1 | 1 | — | — | | 1 | | — | — |
| W. Reg. | — | 2 | 4 | 3 | — | 6 | | 8 | 5 | 20 | 30 |
| T. V. | 12 | 5 | 4 | 2 | 6 | 5 | | — | — | — | 1 |
| L. V. | — | — | 1 | 2 | 1 | 12 | | 180 | 240 | 660 | 240 |
| R. v. L. | 2 | — | — | — | — | — | | — | — | — | — |
| J. Sch. | 2 | 1 | 20 | 40 | 90 | 90 | | 20 | 30 | 72 | 48 |
| C. V. | | — | — | — | — | — | | — | — | — | — |

Table IV — Parasite counts (number of parasites per 6000 leuc

| | April 1941 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 28 | 29 | 30 | 1 | 2 | 3 | 4 | 5 |
| T. S. | — | — | — | — | — | — | | — |
| A. v. D. | — | — | — | — | — | — | | — |
| D. de R. | 1 | 2 | — | — | 1 | | | 12 |
| W. de R. *) | 90 | 30 | 150 | 180 | 240 | 150 | | 180 |
| S. F. | — | — | 1 | — | — | — | | — |
| J. T. | — | 2 | 1 | 1 | — | — | | — |
| J. Kl. | 1 | — | 10 | — | 4 | — | | 2 |
| W. v. Z. | — | — | — | — | — | — | | |
| Adr. v. Z. | — | — | — | — | — | — | | 2 |
| W. Reg. | — | — | — | — | — | — | | — |
| T. V. | — | — | 1 | — | — | — | | — |
| L. V. | — | — | 3 | 12 | 8 | 12 | | 15 |
| R. v. L. | 3 | — | 3 | 5 | 10 | | | — |
| J. Sch. | — | 1 | 1 | — | 1 | 1 | | — |
| C. V. | — | — | — | — | — | — | | — |

Table V — Parasite counts (number of parasites per 6000 leuc

| | June 1941 | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| T. S. | — | — | — | — | — | — | |
| A. v. D. | — | — | — | — | — | — | |
| D. de R. | — | — | — | — | — | — | |
| W. de R. *) | 132 | 150 | 84 | 120 | 96 | 72 | |
| S. F. | — | — | — | — | — | — | |
| J. T. | 15 | 40 | 6 | 4 | — | — | |
| J. Kl. | 3 | — | — | — | — | — | |
| W. v. Z. | 30 | 90 | 270 | 144 | 420 | 360 | |
| Adr. v. Z. | — | — | — | — | — | — | |
| W. Reg. | — | — | — | — | — | — | |
| T. V. | — | — | — | — | — | — | |
| L. V. | — | — | — | — | — | — | |
| R. v. L. | — | — | — | 1 | 4 | — | |
| J. Sch. | — | — | — | — | — | — | |
| C. V. | — | — | — | — | — | — | |

*) Spleenless carrier.
— No parasites found.

ood of 15 healthy carriers in January and February 1941.

| | February 1941 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| | — | — | — | — | — | 1 | | — | — | — | | | |
| | 180 | 150 | 360 | 360 | 240 | 90 | | 2 | — | — | | | |
| | 360 | 600 | 360 | 60 | 30 | 1 | | — | — | | — | | |
| | 240 | 15 | 60 | 48 | 48 | 40 | | | 96 | | 270 | | |
| | 2 | — | 1 | — | 2 | 10 | | | 150 | 300 | | | |
| | 1 | 8 | — | — | — | — | | — | — | — | | | |
| | 6 | 8 | | — | 5 | — | | 4 | — | | — | | |
| | — | — | — | — | 1 | 3 | | 15 | 40 | 60 | | | |
| | 1 | 7 | 4 | 4 | 6 | 7 | | 25 | 10 | 10 | | | |
| | 50 | 30 | 60 | 60 | 96 | 120 | | | 120 | 90 | | | |
| | — | — | — | — | — | 1 | | 1 | | 1 | | | |
| | 40 | 48 | 25 | 3 | 3 | — | | | | 35 | | | |
| | 20 | 15 | 35 | 60 | 150 | 25 | | 4 | 1 | | 4 | | |
| | 1 | — | 2 | 1 | | 1 | | | | 8 | | | |
| | — | — | — | — | — | — | | | — | | — | | |

lood of 15 healthy carriers in April and May 1941.

| 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| — | — | — | | — | — | — | — | — | — | | — | — | — |
| — | — | — | | — | — | — | — | — | — | | — | — | — |
| 4 | 1 | — | | — | — | — | — | — | — | | — | — | — |
| 120 | 40 | 30 | | 30 | 21 | 20 | 25 | 25 | 9 | | 20 | 21 | 40 |
| — | — | — | | — | — | — | — | — | — | | — | — | — |
| — | — | — | | — | — | — | — | — | — | | — | — | — |
| 3 | 1 | — | | 2 | — | — | | — | — | | — | — | — |
| 3 | 3 | 3 | | 45 | 30 | 60 | 78 | 84 | 36 | | 9 | — | 1 |
| — | — | — | | — | — | — | — | — | — | | — | — | — |
| — | — | — | | 8 | 30 | 3 | 20 | 2 | 1 | | — | — | — |
| — | — | — | | — | — | — | — | — | | | — | 1 | — |
| 2 | 4 | 4 | | 1 | — | — | — | — | — | | — | — | 1 |
| 6 | 3 | 4 | | 6 | 6 | 6 | 1 | 3 | — | | 1 | — | 3 |
| — | — | | | 1 | — | — | | — | — | | 1 | 1 | 3 |
| — | — | — | | — | — | — | — | | — | | — | — | — |

blood of 15 healthy carriers in June and July 1941.

| | | | | | | | | | | | | July 1941. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 1 |
| 1 | — | — | — | | — | — | — | — | — | — | | 1 | — |
| — | | — | — | | — | 5 | — | — | — | — | | — | — |
| — | — | — | — | | — | — | — | — | — | — | | — | — |
| 102 | 25 | 15 | 40 | | 90 | 30 | 45 | 72 | 60 | 204 | | 210 | 108 |
| — | — | — | — | | — | — | — | — | — | — | | — | — |
| 2 | — | 2 | — | | — | 1 | 1 | — | — | — | | — | — |
| — | 2 | — | — | | — | — | 2 | 1 | | 2 | | 1 | 1 |
| 2 | — | — | — | | 1 | — | — | 2 | 2 | 1 | | — | 6 |
| — | — | — | — | | — | — | — | | — | — | | — | — |
| 2 | 1 | 22 | 42 | | 156 | 138 | 132 | 84 | 7 | 5 | | — | — |
| — | — | — | — | | — | — | — | — | — | — | | 1 | — |
| — | — | 1 | | | 1 | 6 | 9 | 8 | 2 | 7 | | 5 | — |
| 1 | 1 | 3 | 1 | | 5 | 2 | 3 | — | 10 | 1 | | 2 | — |
| — | — | 1 | — | | — | — | — | — | — | — | | — | — |
| — | — | — | — | | — | — | — | — | — | — | | — | — |

initially increased (parasite relapse) and subsequently dropped down to zero again (see fig. 1—3).

Among the 6 exceptions 5 children occurred, who as early as January had got completely the better of their parasites (test usually negative) and a spleenless boy, whose case will be discussed later on (test always positive).

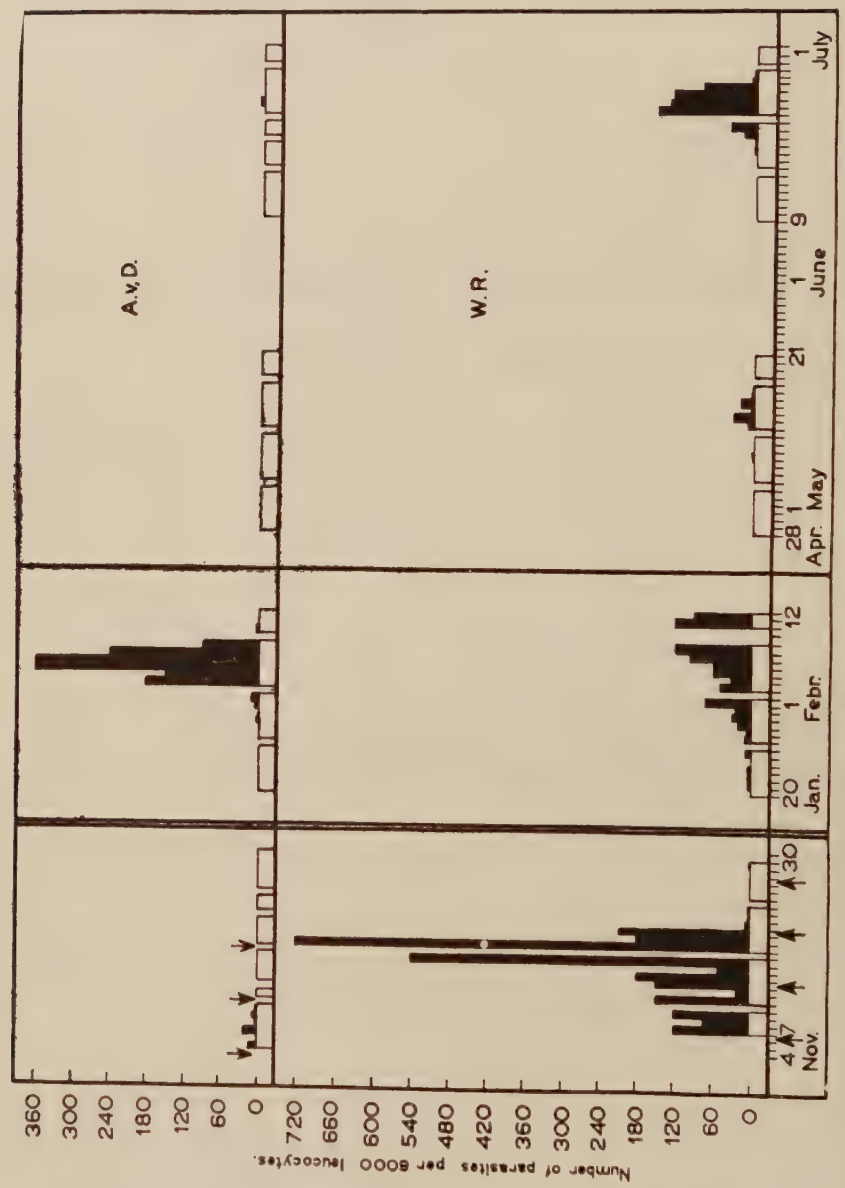



Fig. 1. Healthy carriers (A. v. D. and W. R.) during four periods of 3½ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

The length of the periods in which an uninterrupted series of positive results was obtained ('positive phases') varied in January/February between 11 and 16 days (11, 12, 13, 14 and 16 days; average 13 days), in April/May between 6 and 13 days (6, 8, 12 and 13 days; average 10 days) and in June/July between 9 and 11 days (average 10 days). So the 'positive periods' tended to grow

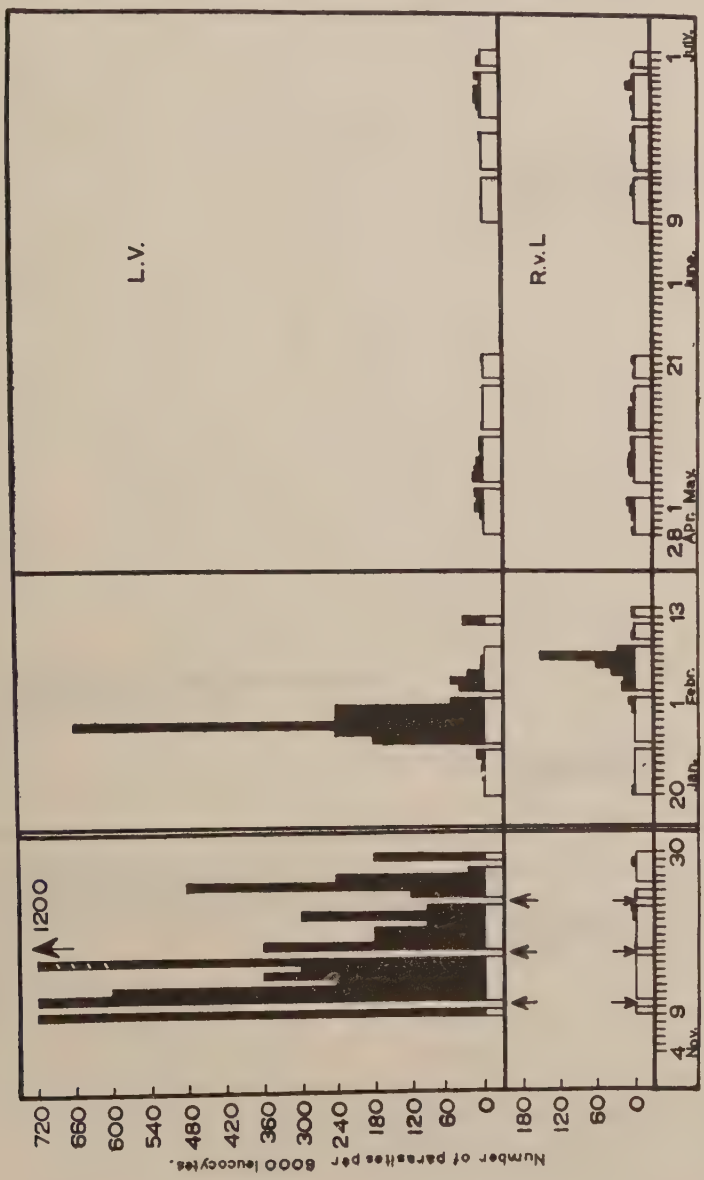
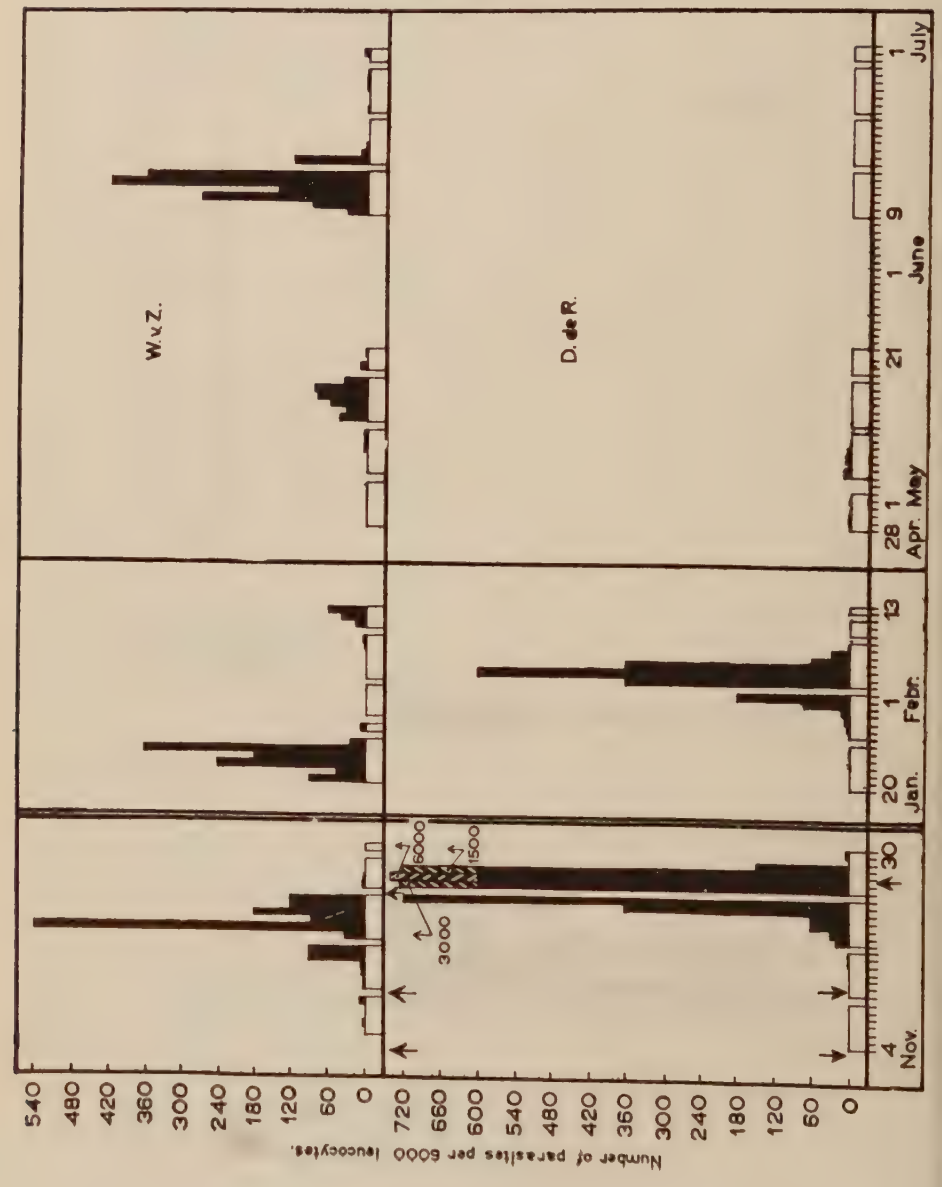



Fig. 2. Healthy carriers (L. V. and R. v. L.) during four periods of 3½ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

shorter during the period of observation. Moreover, the tops came down gradually to a lower level; in two carriers only (W. v. Z. and W.R.), the data of whom are represented in the graphs, we noted higher numbers in June than in May.

During the period of general decrease in number of the circulating parasites between January and July, the peripheral blood of several



carriers gradually grows free from parasites. So out of 9 carriers, in whom in January still fairly significant numbers of parasites had been noted, showing, moreover, the alternating '+' and '—' phases (the spleenless boy thus left out of consideration) in May 7 and in June only 6 were left. In one of these (R. v. L.) as early as May the periodicity had changed into a practically continuous presence of very few parasites (1—10 per 6000 leucocytes).

The length of a 'negative phase' is known to us from the January/February series only. In three carriers we could note in the beginning of the observation period of $3\frac{1}{2}$ weeks the ending of a '+ wave' and, at the close of the period, the beginning of a new one. The duration of the 'negative' intervals was 8, 10 and 14 days (average 11 days). In the later series never any 'negative phase' limited to a period of $3\frac{1}{2}$ weeks could be noted. The chance of its occurrence may well have been diminished by the already mentioned decrease in number of the carriers. We can hardly surmise, however, that this would be the only reason for the non-occurrence of complete 'negative phases' in May and June, as in this period several positive periods did occur. So we are inclined to suppose that between January and July, along with the slight shortening of the '+' phases, the '—' phases have increased in length; as a matter of fact this will diminish the chance of noting its beginning and its end in one and the same observation period. Whether an inverted ratio exists between the length of the negative and positive phases, *i.e.*, whether the intervals between the tops of the '+' phases are of equal length, is an interesting question, which, however, cannot be answered without further investigation.

With regard to epidemiological investigation these facts show that (at least between January and July) the actual number of parasite carriers in a population-group can never be discovered by preparing a single blood film for each member, because part of the members of such a group will just be in a 'negative phase'. Table VI D shows the percentage of the actual carriers which would have been traced in our series by means of a single test.

Table VI

| | Jan./Febr. | April/May | June/July |
|---|---|---|---|
| A. average number of parasites (per 6000 leucocytes) counted during the positive phases | 62 | 22 | 46 |
| B. maximum number of parasites per 6000 leucocytes | 660 | 270 | 270 |
| C. % positive results | 60 | 36 | 30 |
| D. % of the carriers which would have been traced in one day | 61 | 40 | 36 |

Daily examination of all concerned would be far too laborious for an investigation in the epidemiological field. In accordance with the presented data on the periodicity of the parasite relapses, it may, however, be claimed as sufficient to carry out a second and a third blood test about 10 and 16 days after the initial one. By these means, only such carriers will escape their being traced by the investigator in whom, fairly rarely, slight numbers of parasites can be detected and who as such are of little significance for the final result.

As in the various carriers the tops do not occur simultaneously, changing metereological conditions cannot provide an explanation for the described periodicity. It might be considered, however, as an image of the struggle between parasite and host. After each revival of the activity of the parasites in the reticulo-endothelial tissue, (which may give rise to a 'parasite relapse' in the periphery) a certain reserve of specific counteracting force seems to have originated (negative phase). In the course of a limited number of days, however, this defensive reaction decreases in such a measure, that the parasites may come into play again. After some months the periodically revived activity of the parasites in their hiding-places has apparently weakened in such a degree that it can only manifest itself in the peripheral circulation, when it is at its h e i g h t : then during a few days a small number of parasites will occur in the blood (short and low '+' wave).

### A spleenless carrier of parasites

Among the six carriers in whom the periodical appearance and disappearance of the parasites was less distinct than in the others occurred a boy of eight years (W. de R.) whose spleen had been removed in March 1938 on account of a trauma. In May 1938 he had his first attack of malaria. Thus he had probably been infected in autumn 1937 and may well have been a carrier of parasites since May 1938, as he had since been complaining of many accesses of fever. If we leave the November series (during the quininisation) out of account for the moment, we note that in him the result of the blood test has n e v e r b e e n n e g a t i v e (fig. 4). It is true that the curve for the numbers of parasites in his blood shows distinct rises and falls, but whilst for other carriers the 'waves' from January to July generally either decrease markedly in height or disappear completely and at the end of each decrease in number the zero line is reached again, in the spleenless boy the imaginary line which would connect the tops of the 'waves' descends but very slowly and every day parasites could be detected in his blood.

The fact that for him the tops in January were of about the same height as for the other carriers may not be considered as of much value. Indeed the spleenless boy had probably been carrier during a much longer period than most of his companions in infortune.

When in November 1941 in all 15 carriers one more blood test

was made, the spleenless boy was one of the two in whom parasites were traced and even in a fairly high number(100/6000 leucocytes). Whilst many had vanquished their germs completely — or nearly so — in July (*viz.*, in most of the cases about a year after their initial attack of malaria), the spleenless boy had not yet attained this stage in probably $3\frac{1}{2}$ years after the first access of malarial fever. We need not wonder at this, as it is well known, that the spleen is specially active in destructing malaria parasites. According to TALIAFERRO(5) it is, moreover, the field of action of a specific antibody, causing an opsonic activation of macrophagocytes.

The fact, that even in the absence of the spleen the tops of the curves do not surpass a definite height in the end, shows that even under these conditions the body may defend itself against the parasites and eventually free itself from them (*e.g.* by means of the remaining part of the reticulo-endothelialsystem).

It was, moreover, remarkable that during the autumnal quininisation the blood test in the spleenless boy remained negative for a long time without interruption. This does neither agree with results, obtained in him in later series, nor with those in most of the other carriers of parasites in the period in which they took quinine. Possibly parasites occurring in the spleen are less vulnerable for quinine than those which are hiding in other organs, *e.g.* in the bone-marrow or in the liver. Another possibility lays in the assumption that the circulating parasites, which are damaged prima-

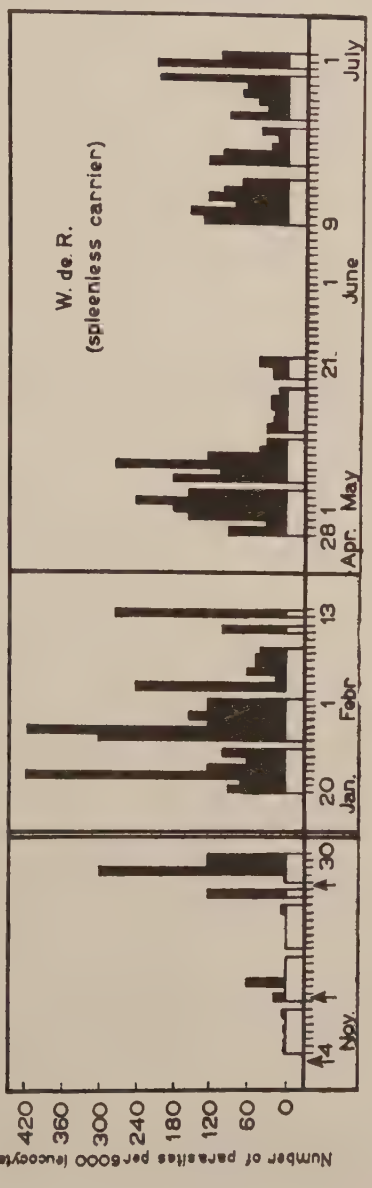



Fig. 4. A spleenless carrier (W. de R.) during four periods of $3\frac{1}{2}$ weeks. The white rectangles enclose the days at which a blood count was made. The arrows point to the days when quinine was prescribed.

rily by the quinine, are more swiftly replaced in a person with a larger store of parasites.

### III Stages of development of the parasites.

All forms, also gametocytes (especially male) occurred up till the end of the observation period. No change in the ratio of their occurrence could be noted.

The tertian periodicity remained recognisable and was most distinct in the April/May series, in which as a rule we noted during the '+' phase every other day an alternation of mostly young with mostly full grown rings. As, moreover, the schizonts broke into a normal number of merozoites, it may be supposed that the reproductional process is not influenced disadvantageously by the defensive action of the carrier. Other investigators are of the same opinion (cf. Taliaferro (5)).

### IV The state of health of the parasite carriers.

Our observations remained restricted (i.a. by the insufficient cooperation of those concerned) to the subjective health statements, the body temperature and the report of the school physician.

### The connection between the seriousness of the complaints and the number of parasites in the blood

1. In carriers of parasites who felt in perfectly good health and complained at most sometimes of a headache, we counted 960, 2400 and 2520 parasites per 6000 leucocytes.

2. 'Seedyness' and not visiting school (without calling in medical advice) we noted to occur along with 180, 3600, 4500 and 6060 parasites per 6000 leucocytes.

3. Carriers who felt ill and who, notwithstanding this, did not seek medical advice and took quinine on their own authority had 3900, 4920, 6000, 9000 and 9030 parasites per 6000 leucocytes in their blood. Sufferers from malaria with as high a number of parasites thus may remain untraced, since as a rule they do not seek medical advice even in a relapse, as long as they are still provided with quinine. Moreover, such a treatment with quinine on their own initiative mostly lasted for a few days only! As most of the numbers mentioned here bear on autumn and as this is also the season in which the mosquitoes transmit the malaria parasites, it is not to be wondered at that the disease in the population group observed by us has maintained itself over a long period.

4. When they felt ill in such a degree that medical advice was sought for, the numbers of parasites varied between 6000 and 24000 per 6000 leucocytes.

## The body temperature of the carriers

In the summer of 1940 the blood of several carriers was tested daily during 7 to 10 days and as far as possible their temperature, taken rectally, registered. When more than 240 parasites per 6000 leucocytes were counted, a rise of temperature was always noted; lower numbers often occurred along with a perfectly normal temperature. On the other hand we could note twice a rise in temperature up till 38° C. during a short period, whilst not a single parasite could be traced in the peripheral circulation; 2 or 3 days after the rise in temperature, however, parasites occurred again in the blood in both cases and subsequently increased greatly in number.

## The report of the school physician

From none of the children concerned special complaints had come in during the period of our observation.

In fact the sufferer from chronic malaria escapes in a number of ways the attention of his surroundings and of those whose task it is to combat the disease. Not only any typical symptoms are usually absent, but, moreover, the result of the blood test is often over a long period negative and many complaints, even those of a fairly serious character, do not reach the physician. As a consequence, the term 'healthy carrier' may not be entirely justified, but it is clear now, why it has become a generally accepted one in the course of time.

## Summary

In 1940 and 1941 observations were made on the number and mode of appearance of parasites of benign tertian in the blood of 'healthy' carriers of parasites. The results of this investigation indicate that during the period of incubation shortly before the attack no or at most a few parasites occur in the peripheral circulation; this agrees with the experience of others.

The group of carriers who had already suffered from an attack was studied; in this country these are the main source of infection for the mosquitoes. It appeared — at least between January and April following the year in which they had fallen ill — that as a rule periods in which the blood test is negative alternate regularly with others in which the number of parasites first increases (parasite relapse) and subsequently drops down to zero again. The length of the negative and that of the positive phase averages about 12 days.

No parasites — or only a few of them — are to be found in the blood of most carriers one year after the outbreak of the disease. This was not the case in a spleenless carrier even after 3½ year; moreover, the blood test of the latter was never negative.

The tertian periodicity remained recognisable up till May 1941.

Some data were collected on the general state of health and the body temperature of the carriers. It may happen that a person with 2400 parasites/6000 leucocytes still has the impression of being in quite good health.

An attempt to induce the disappearance of the parasites from the peripheral circulation by means of autumnal quininisation in a population group is described; the results, however, were disappointing.

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(From the Laboratory for Hygiene, Bacteriology and Tropical Hygiene of the University, Leiden)

# VIBRIO RESEARCH IN THE HEJAZ IN CONNECTION WITH THE EL TOR PROBLEM

by

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(Received March 25, 1944).

## 1. The history of the El Tor problem.

It was Robert Koch, who rendered a great service to mankind in discovering *V. cholerae* as the originator of the cholera asiatica in 1883, which fact offered the possibility to take appropriate hygienic and prophylactic measures against this very infectious disease. He succeeded in cultivating vibrios out of the stools of patients suffering from cholera and out of those of people, in whom the disease proved fatal. These micro-organisms proved morphologically, culturally as well as serologically specific. He also stated, that people by drinking water containing a large amount of these vibrios might be infected by the disease.

Later on investigators of many countries could isolate out of the water of numerous rivers, wells and sources vibrios, which differed in some respects from the cholera vibrio of Koch and which therefore were reckoned to the group of water vibrios.

Moreover during many epidemics of cholera out of different surface waters vibrios were cultivated by means of the enrichment method of Schottelius with peptone water. By means of the methods of research then available these vibrios could not be distinguished from *V. cholerae*, and so were qualified as such (24).

In those days only pathogenic *V. cholerae* and apathogenic water vibrios were known, organisms which by means of the bacteriological methods (biochemical test, agglutinating reaction of Gruber and Durham, Pfeiffer's test) could be easily differentiated.

In later years cholera-like vibrios were isolated out of human feces, which were morphologically and culturally similar to *V. cholerae* but which were not agglutinated by highly agglutinating cholera serum (3).

In 1897 on a pilgrimship Ruffer cultivated vibrios out of the stools of a pilgrim returning from Mecca and who suddenly died; these bacteria were found to be quite similar to *V. cholerae*, although in the Hejaz, neither on the ship nor at the quarantine station El Tor cholera occurred.

So during some years for all returning pilgrims from Mecca, who

had died at this quarantine station, it was investigated whether vibrios occurred and autopsies were carried out on all corpses. This resulted in the isolation during five successive pilgrim seasons of many vibrios, which by means of the agglutinating test could not be distinguished from *V. cholerae* (26).

In 1905 GOTSCHLICH detected vibrios in 38 out of 107 people returning from Mecca, 6 of which were agglutinated by highly agglutinating cholera serum and 32 not. These vibrios were considered by GOTSCHLICH as real cholera vibrios, although there was no cholera in the Hejaz, neither on the pilgrimship nor at the quarantine station El Tor. The people in whom the vibrios were found were qualified by him as carriers of cholera vibrios (9).

RUFFER, who was president of the „Conseil sanitaire maritime et quarantenaire d'Egypte" did not accept this diagnosis and declared the 6 agglutinable vibrios as not identic with *V. cholerae*. He was of opinion, that the agglutination test was useful but not specific and a conclusion as to the specific nature of these El Tor vibrios might merely be arrived at after the problem had been viewed epidemiologically as well as bacteriologically. Because of the fact that the 6 pilgrims in whom the El Tor vibrios had been found had left their country already 2 or 3 months earlier and as cholera vibrios after about 2 weeks will have disappeared from the intestines of a patient (CRENDIROPOULO 1902), RUFFER thought it probable, that the pilgrims might have been infected with the vibrios at Mecca. Although RUFFER had only found inagglutinable vibrios and no agglutinable ones in the Zam-Zam water from Mecca, he supposed that this water, which is drunk by all pilgrims, was the source of origin of *Vibrio El Tor* (26).

So the El Tor problem had arisen, *viz.*, the problem whether *V. El Tor* is identic with *V. cholerae* (monistic point of view) or not (dualistic conception).

This question is not solved as yet; some investigators are of opinion that both vibrios are identic, as they have the same serological properties, others regard them as two kinds of vibrios, as they differ in action on blood plates and in blood broth and as moreover in recent years differences in the chemical structure of the two bacteria have been stated.

KOLLE and MEINICKE were the first investigators, who in 1906 supported the opinion of GOTSCHLICH (12).

In the same year KRAUS and PRIBRAM opposed the monistic conception for epidemiological reasons and because of the fact, that *V. El Tor* produced haemotoxine in blood containing media whilst *V. cholerae* lacked this property (13).

Later on MÜHLENS and VON RAVEN (1906) (23), NEUFELD and HAENDEL (1907), BAERTHLEIN (1914) (1), CLAUBERG (1921), MACKIE (1929) agreed with the monistic point of view; on the other hand LIEFMAN and NIETER (1906), KRAUS and TRANTSCHOFF (1906), KRAUS and RUSS (1908), KRAUS and FUKUHARA (1909), PFEIFFER (1908), VAN LOGHEM (1910) (18), GRAIG (1914), LÖWY (1915) (15),

KÄMMERER (1920), LAMPE (1922) (13) defended the dualistic conception.

In collaboration with STEENSMA (17) and later on with SNAPPER in 1918 (27) VAN LOGHEM ascertained the haemolytic action of *V. El Tor* in contrast with the haemodigestic property of *V. cholerae*.

As according to several investigators the haemolytic property was not only found for *V. El Tor* but also for *V. cholerae*, in 1922 LAMPE (14) ascertained that the *V. El Tor* haemolysine was formed very soon, that it was partly thermolabile and partly thermostabile and that it had an antigenic action (exohaemolysine), whilst the cholera haemolysine was formed later and had no antigenic character (endohaemolysine).

In 1931 once more some *V. El Tor* strains out of the feces of pilgrims not suffering from cholera were isolated by DOORENBOS. He is the only investigator who considered the *V. El Tor* as a *V. cholerae* infected with bacteriophage, so he is a supporter of the monistic theory (2).

VAN LOGHEM in 1933 pointed out emphatically the diagnostic value of properties of haemolysis of *V. El Tor* and of haemodigestion of *V. cholerae* (19).

In the same year FLU examined some cholera and El Tor strains biochemically and serologically. In these experiments 5 cholera strains, which 16 years earlier had shown no haemolytic action, now appeared to possess an although temporary haemolytic property. Because of this reason FLU, who was formerly supporter of the dualistic point of view, began to doubt it (5).

Meanwhile new serological methods of investigation were adopted and several investigators as BALTEANU (1926), SHOUSHA (1931), ZIMMERMANN (1932) (29), ABDOOSH (1932), GOHAR (1932), GARDNER and VENKATRAMAN (1935) (6), considered only the cholera O-serum as specific; but even using this specific cholera O-serum none of them except GOHAR was able to distinguish *V. El Tor* from *V. cholerae*.

According to GISPEN in 1938 the agglutinating property of *V. El Tor* would be more heat-stable than that of *V. cholerae* (8).

Other investigators tried to divide the vibrios according to their biochemical reactions or to their chemical structure.

HEIBERG examined in 1935 more than 400 vibrio strains by applying mannose, saccharose and arabinose media; so he arrived at 6 groups of vibrios, but *V. El Tor* and *V. cholerae* appeared to belong to the same group (10).

In 1932 LINTON and his collaborators analysed the vibrios chemically and found that they contained several combinations of 3 polysaccharides and 3 proteins; in this way he was able to classify the vibrios into 6 groups and so to distinguish *V. El Tor* from *V. cholerae* (16).

In the meantime in 1937—1938 an explosion of gastroenteritis

broke out in South Celebes (Macassar and its surroundings), which according to the examination of DE MOOR was caused by vibrios, which were in every respect quite similar to *V. El Tor*. The same vibrios were cultivated out of waters, which were probably polluted with the stools of the patients (21). The correctness of this diagnosis was confirmed at VAN LOGHEM's laboratory at Amsterdam. This was the first time, that *V. El Tor* was stated as the originator of a disease which resembled the cholera; this fact gave a new turn to the El Tor problem (20).

2. RESEARCH IN THE HEJAZ.

DE VOGEL, delegate of the government of Netherlands East India and member of the „Comité de l'Office international d'hygiène publique", suggested in 1935 that the Dutch government might have the possibility investigated of the transmitting of El Tor vibrios to and from Mecca. For this purpose control ought to be carried out in the countries of origin of the pilgrims and in the Hejaz itself.

In Netherlands East India this investigation was carried out by MEYER (22), whilst in the Hejaz the task was given to me.

This control was carried on from October 1937 till June 1938 along with my own extensive clinical work and in very difficult circumstances. During 9 months I examined 1109 stools of different people and 90 water samples out of various water sources. In view of the El Tor gastroenteritis in South Celebes special attention was paid to the pilgrims coming from this part of India.

The stools of healthy and sick people coming at the policlinic were controlled, namely 602 pilgrims from Netherlands East India, 310 Indonesians living at Mecca, 21 pilgrims from the Malay States, 113 Malays living at Mecca and 63 Arabs and people of other nationalities.

The control of the feces and the waters was done by means of the enrichment method of SCHOTTELIUS with the Dieudonné blood plate and further by applying the common bacteriological tests.

Vibrios were isolated out of the stools of 11 persons, 2 of whom were pilgrims freshly arrived (1 from South Celebes), 7 were Indonesians from Mecca and 2 were negroes living as well since a long time in this country. These vibrios were motile, Gram negative, they fermented glucose, maltose, mannose and saccharose, did not ferment lactose, formed a bright pink surrounding area on the goats blood plate, haemolysis in goat blood broth, gave a positive cholera-red test and were agglutinated by cholera-OH-serum till a titre varying from 1 : 400—1 : 3200.

According to a further investigation at Amsterdam and at Batavia they were not agglutinated by cholera-O-serum and therefore were neither *V. cholerae* nor *V. El Tor*.

In connection with the water control as to the occurrence of vibrios some information about the water supplies in the Hejaz may be presented.

The people in this part of Arabia derive their drinking- and

bathing-water only from the few wells and sources in the oasis and in the lower parts of the country and by gathering the rarely falling rainwater. Most of those wells exist since hundreds of years and some of them are regarded by the inhabitants as holy. The most important and holy well is the Zam-Zam well in the central mosque at Mecca, the water of which is drunk by all pilgrims coming there. The Ain Zoebaida for Mecca is the longest and the best waterchannel, it has been built about 700 years ago on order of queen Zoebaida. In more recent years at the harbourtown of Djedda condensed sea water is also to be had, which is the best drinking water in this town.

I examined most of the water sources in the Hejaz as to the occurrence of vibrios namely:

| | | |
|---|---|---|
| 29 samples | Zam-Zam water | } Mecca |
| 9 samples | Zoebaida-channel water | |
| 3 samples | Wasirya-channel water, Djedda | |
| 4 samples | Sarga channel water, Medina | |
| 45 samples | out of the other water wells and sources | |
| 90 samples | totally | |

Van Romburgh in 1886 had already examined chemically the Zam-Zam water which he had obtained from Snouck Hurgronje.

This water was clear, contained no ammonia and a negligible quantity of organic substance (25). The chemical composition of 1 liter of this water was the following:

| | | |
|---|---|---|
| Cl | 0.5563 | gram |
| $SO_3$ | 0.3955 | ,, |
| $N_2O_5$ | 0.7255 | ,, |
| $CO_2$ | 0.3170 | ,, |
| $SiO_2$ | 0.4720 | ,, |
| CO | 0.4120 | ,, |
| MgO | 0.1520 | ,, |
| $Na_2O$ | 0.5776 | ,, |
| $K_2O$ | 0.2719 | ,, |

A second examination of 0.25 l of the water gave the following results:

| | | |
|---|---|---|
| $CaSO_4$ | 0.2501 | gram |
| $MgSO_4$ | 0.1140 | ,, |
| $Na_2SO_4$ | 0.3307 | ,, |
| $K_2SO_4$ | 0.1257 | ,, |
| $SiO_2$ | 0.0118 | ,, |
| Totally | 0.8323 | gram residu |

Moreover the water had a particular metallic taste, which is known to me by own experience.

According to some European travellers, such as Burton and Burckhardt in 1829 and also according to the inhabitants of Mecca the Zam-Zam water has a laxative action, when drunk in a large quantity.

The first investigator who has examined the water bacteriologically was RUFFER in 1907 (see above).

In 1928 VAN DER HOOG stated, that the Zam-Zam water did not contain ammonia and nitrates but only nitrites; the coli fermenting test was positive in 5 cc, the number of bacteria was 1880 in 1 cc (11).

From 12 out of the 29 samples of Zam-Zam water I cultivated vibrio strains, one of which appeared to be identic with *V. El Tor*, according to the examination at Amsterdam as well as at Batavia.

The 11 other strains were agglutinated by cholera-OH-serum but not by cholera-O-serum; therefore they were neither *V. cholerae* nor *V. El Tor* and merely water vibrios.

Out of the 61 water samples derived from the other wells, sources and channels only 4 vibrio strains were isolated, which also appeared to be water vibrios.

So the results of my examinations of stools and water samples in the Hejaz were, that several times water vibrios were found, especially in the Zam-Zam water and that once a vibrio strain was cultivated, which was in every respect identic with *V. El Tor.*

This investigation was carried out in a period when no cholera occurred in the Hejaz. Thousands of pilgrims had drunk the Zam-Zam water without any harmful effect.

3. RESEARCHES ON VIBRIO CARRIED OUT BY OTHER INVESTIGATORS.

The results of MEYER's investigation in 1936 at Batavia for 20444 persons and those of SARDJITO at Semarang in the same year for 2875 people and 40 water samples as to the occurrence of *V. El Tor* were negative (22).

Neither in Egypt was *V. El Tor* found, either in stools or in waters (3).

After GOTSCHLICH in 1905 and DOORENBOS in 1930, the latter isolated again in 1931, 1932, 1933 and 1934 at El Tor, El Tor vibrios out of the stools of home-bound pilgrims (4).

In 1933 SHAHIN PASCHA examined 1532 Egyptian pilgrims on their outward as well as on their homeward voyage. Whilst no *V. El Tor* was found before they went, it appeared that home-bound 8 of them carried these vibrios.

SUHBI WAHBI in 1938 examined home-bound Irak pilgrims at the landquarantine station Najaf and found in the feces of 1 out of 61 persons El Tor vibrios (28) and according to GILMOUR in 1938 there were found 8 El Tor vibrio carriers among the home-bound Egyptian pilgrims (7).

## Summary and conclusions.

In 1883 ROBERT KOCH discovered *V. cholerae* as originator of the cholera asiatica; this important discovery has enabled us to conquer the very infectious and dangerous disease. Subsequently water vibrios were found in many water sources; these microbes

can easily be differentiated from *V. cholerae*. Several times *V. cholerae*-like vibrios were isolated out of the stools of people suffering from gastroenteritis.

In 1905 GOTSCHLICH and afterwards other investigators as well isolated at El Tor out of the feces of pilgrims who were not suffering from cholera, vibrios which according to some of them were identic with the actual *V. cholerae*, but which in the opinion of others such as RUFFER must not be considered as such.

DE MOOR cultivated in 1937—1938 at Macassar and its surroundings out of the stools of people suffering from a cholera-like gastroenteritis as well as out of waters, which were probably polluted with the feces of those people, germs which in every respect were like the El Tor vibrio.

Finally I detected in 1937—1938 in a water sample derived from the Zam-Zam well at Mecca (Hejaz), the water of which is drunk by thousands of pilgrims without any harmful effect, germs which were absolutely identic with *V. El Tor* of GOTSCHLICH.

The El Tor vibrio is never found in the stools of people bound for the Hejaz, but several times in the feces of home-bound pilgrims.

These facts make me tend to the following conclusion:

I. *V. El Tor* forms a group of vibrios of its own, neither identic with *V. cholerae* nor with the water vibrios.

II. This group of vibrios may be subdivided into 2 types:
1°. The apathogenic type, which is found in the Hejaz and surroundings; this vibrio may be called: *Vibrio El Tor*, type Hejaz.
2°. The pathogenic type, which is found at Macassar and surroundings: this may be called: *Vibrio El Tor*; type Macassar.

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(From the Microbiological Laboratory of the Agricultural University, Wageningen).

# THE ACTION OF MANGANESE ON THE DEVELOPMENT OF *ASPERGILLUS NIGER*

by

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## 1. Introduction.

The reason why an attempt has been made to compose a manganese standard with *Aspergillus niger* as indicator is the following.

A plant disease exists, known in this country as „Veenkoloniale haverziekte" (grey-speck disease, Dörrfleckenkrankheit), which is apt to occur on sandy humous soils as the result of too strongly alkaline manuring. As oats are particularly sensitive for this disease, this crop plant has been the first in which its symptoms have been noted (Sjollema and Hudig (20)). Most markedly under influence of a sudden raise in temperature the leaves will present greyish dry spots and the tip parts of the leaves will fall over with a sharp kink at the necrotic region. The further development will stagnate and the ultimate yield will be influenced very unfavourably. An acid manuring will prevent the further occurrence of this disease, but a more direct although not lasting result may be attained by a dressing with mangano sulphate or other manganese compound.

So the question arose how to explain the favourable action of manganese.

The fact that manganese might range among the elements essential for the normal development of plants has been suspected already a long time ago. In 1909–12 Bertrand (1, 2, 3) has pointed to the beneficent effect on plant development of small gifts of manganese to the soil. Mazé (13) working with corn plants in highly purified solutions notes a weaker development and a chlorose in the leaves in the absence of manganese. McHargue (14) could induce definite deficiency symptoms in the foliage of peas, when grown in a solution deprived of manganese. An important investigation of also distinct agricultural value was carried out by Samuel and Piper (19) in South Australia. These scientists succeeded in inducing in oats, grown in culture solution made up with very pure chemicals and free from any manganese, symptoms of a similar nature as those of the grey-speck disease. When, however, 0.25 mg per liter had been added to the solution, healthy

plants developed. When the content of Mn in healthy and diseased plants grown in the field was compared, it was in the latter markedly lower than in the former; whenever the content fell below a definite level the symptoms of the disease were apt to occur. So the connection between the presence of plant-available manganese and a healthy plant development has been well-established. More recent research may have shown that the connection is of a more complicated nature than originally surmised (GERRETSEN (8)), the fact that manganese is an essential element is left unshaken.

It has been tried along several lines to estimate the content of plant-available manganese in the soil. For a recent survey of the literature on this subject the reader is referred to WIERINGA (28).

As has been stated already, grey-speck disease occurs mainly in alkaline soils not below pH 6.7. Manganic compounds vary greatly in solubility, depending on the presence either of mangan*i* or mangan*o* ions. For the ascertainment of plant-available Mn the determination of the total amount in alkaline soils is of no value. PIPER (18) arrives at the conclusion, that its solubility will depend: 1° on the pH of the soil: under alkaline condition mangan*o* ions being oxidised to mangan*i* and thus insoluble; 2° on the redox potential: under reducing conditions mangan*i* will be reduced to mangan*o* and so the solubility will increase. Later LEEPER (10) could ascertain the existence of healthy alkaline soils, which even at a high redox potential will furnish a leachate high in Mn when quinol has been added to the leaching solution. For sick alkaline soils, however, the addition of quinol will not involve an increase of Mn in the leachate. Apparently the mangan*i* may exist in various levels of activity; when most active it will oxidise other compounds easily and will be reduced to soluble mangan*o*. As the amount of soluble manganese is in such a large measure dependent on experimental conditions and it is thus difficult to estimate the actual amount available to the plant, it seemed worthwhile to investigate whether *Aspergillus niger* might be made to serve this end.

In fact experimental results of many scientists indicate that *Aspergillus niger* assimilates several elements along similar lines as higher plants. An *Aspergillus* standard is in use for the estimation of the available amount of K (NIKLAS (17)) and P (NIKLAS (17), GERRETSEN and BLUMENDAL (9)); in these cases the yield in weight of the fungus for graded amounts of these substances has been determined and thus a standard composed. By MULDER (15, 16) *Aspergillus niger* has been made to serve the estimation of Cu, the shade of colour of the spores marking here the distinction between the various grades of the scale. For Mg (MULDER (15, 16), SMIT and MULDER (21)) it is the density of growth of sporangia which marks the various grades.

The essentiality of Mn for the full development of *Aspergillus niger* has been ascertained by several investigators. BERTRAND

(4, 5) as early as in 1912 succeeded in composing a culture solution by means of which he could provoke a difference in yield of mycelium between the solution completely lacking in Mn and a solution which had been provided with 0.1 $\gamma$ Mn per liter. To attain this result the chemicals had been very rigidly purified by a threefold recrystallization and the fungus had been grown in quartz vessels. In the absence of manganese no sporangia had developed.

STEINBERG (23) presents in 1935 experimental results attained at in the absence of manganese. He frees his culture solution from traces of heavy metals by means of adsorption on $CaCO_3$. He states: „omission of manganese results in the development of intensely white felts consisting of individual and but partly coalesced colonies. The brittle felt is as a result perforated by numerous holes. Spore formation, moreover, is sharply reduced or even suppressed in the absence of manganese. Suppression of spore formation in cultures containing a limited amount of Mn has been noted at yields 3/4 maximum".

So an attempt to compose a graded scale for the estimation of manganese by means of *Aspergillus niger* might prove to be well-founded.

## 2. EXPERIMENTS.

The experimental technique for the composition of a graded scale is very simple. It consists in the cultivation of *Aspergillus niger* in a complete culture solution, the only varying factor consisting in the amount added of the element studied. When for definite amounts of such an element definite responses in the development of the mould occur, such a range in amounts may serve in its turn as a standard for the estimation of unknown amounts of the element in question.

A culture solution such as it has been used by MULDER was employed. Its composition is:

| A | | B | |
|---|---|---|---|
| 50 | g glucose | 20 | mg $FeCl_3 . 6 H_2O$ |
| 5 | g $KNO_3$ | 20 | mg $ZnSO_4 . 7 H_2O$ |
| 2.5 | g $K_2HPO_4$ | 1 | mg $CuSO_4 . 5 H_2O$ |
| 1 | g $MgSO_4 . 7 H_2O$ | 1,5 | mg $Na_2MoO_4 . 2 H_2O$ |
| 1 | l dist. water | 3 | mg $MnSO_4 . 4 H_2O$ |

The finding of a suitable source of iron offered difficulties. The $FeCl_3$ which had been used by MULDER proved not free from Mn; various other iron salts qualified as of high purity were tested, but no clear-cut deficiency symptoms in the absence of Mn could be provoked. Prof. TENDELOO had the kindness to prepare out of chemicals of the highest grade of purity $FeSO_4 . 7 H_2O$, which served my aim perfectly. I am much endebted to him for this help. So all cultures were provided with 85 mg $FeSO_4 . 7 H_2O$ per liter, equivalent to 20 mg $FeCl_3 . 6 H_2O$.

Initially the culture medium was prepared along the same lines as MULDER did, *viz.*, after the chemicals A had been added 5 g coal (norite) per liter was added, the suspension shaken for 5 minutes, filtered through a Buchner filter and supplied with the chemicals B, the oligopleronts. This purification by means of norite, however, did not serve, like this had proved the case in a study on molybdenum (16). The filtrate proved not free from Mn, the norite apparently setting free some trace of manganese. So the method followed by STEINBERG (24) was adopted. 15 g $CaCO_3$ per liter was added to solution A, which was sterilized during 20 minutes at 120° C. The $CaCO_3$ was filtered of on a Buchner filter and the oligopleronts were added.

1 liter Erlenmeyer flasks of Jena glass, which had been frequently in use, were used as culture vessels and were supplied with 40 ml of the medium. All vessels, when not stated otherwise, were put up in duplo. The chemicals used were of the highest grade of purity available. Before use all glassware was rinsed with 10% $HNO_3$, twice with tapwater and finally with distilled water. The distilled water was prepared in an apparatus out of Jena glass. The strain of *Aspergillus niger* used by MULDER was used throughout. Fresh cultures always served for inoculation. The cultures were inoculated with 5 drops taken from a suspension of spores in distilled water. After incubation at 28° C. during 5 days the development of the fungus was ascertained. When the weight of the yield was to be estimated the mat of fungus was removed out of the flask, rinsed in water and dried at 105° C. The duplos were generally weighed together.

STEINBERG (25) in an investigation on molybdenum has established that this element is essential for the full development of *Aspergillus niger* provided nitrate has been supplied as only source of nitrogen; when, however, an ammonium salt acts as such, no deficiency symptoms occur in the absence of molybdenum. He draws the conclusion that molybdenum may be an essential factor in the reduction process of nitrate to ammonium, in which form the nitrogen will be ultimately assimilated by the fungus.

In the physiology of higher plants BURSTRÖM (7) and LUNDEGAARDH (11) present data for manganese proving that the assimilation of nitrate by roots of oats is suppressed in its absence.

So in view of the possibility that similar phenomena might occur in the case of *Aspergillus niger* I started my experiments by comparing the effect of the absence of Mn in solutions provided either with $KNO_3$ or $NH_4Cl$ in amounts equivalent for N. The results, however, appeared to be quite the opposite of those arrived at in the absence of Mo. Whilst the solution with $NH_4Cl$ without any intentionally added Mn gave rise to white felts quite free from sporangia, the $KNO_3$ solution presented a dense growth of sporangia; visually such a culture could hardly be distinguished from one on a medium provided with Mn.

When the final pH of the culture with $NH_4Cl$ was estimated

by means of Lyphan paper, it appeared to have sunk to 1.6. In the nitrate series the final pH was about 4.5. So the suppression of the growth of sporangia might either be due to the absence of Mn or to the low final pH of the culture solution. When, however, Mn had been added to the solution with $NH_4Cl$, sporangia developed, notwithstanding the pH sank at a same rate: the acidity as such apparently did not prevent their development (Fig. 1). The optimal development differed, however, somewhat from the development under less acid conditions, the sporangia occurring in tufts evenly scattered with uncovered regions between. Moreover when an ammonium salt solution had been buffered by a supply of $CaCO_3$ [1]), sporangia developed quite abundantly in the absence of Mn.

Fig. 1. $(NH_4)_2 SO_4$ [2]) as source of nitrogen.
flask 1: — Mn — $CaCO_3$
flask 2: — Mn + $CaCO_3$ (0.5%)
flask 3: 25$\gamma$ Mn — $CaCO_3$

The suppression of sporangia as a symptom of manganese deficiency could apparently merely be induced under strongly acid conditions.

So obviously the effect of acidification of a nitrate solution had to be tested as well. Phosphoric acid prepared from chemically pure anhydrous $P_2O_5$ was supplied for this purpose. The concen-

[1]) This $CaCO_3$ had been prepared out of chemically pure $CaCl_2$ and a solution of $Na_2CO_3$ which had been purified by adsorption on $CaCO_3$ in the same way as this was done for the nutrient solution.

[2]) $(NH_4)_2SO_4$ and $NH_4Cl$ act similarly.

tration of $H_3PO_4$ actually used was 3.21 n, when titrated with thymolphtaleine as indicator. Whenever in the following volumes of $H_3PO_4$ will be mentioned, this will bear on 3.21 n $H_3PO_4$.

The size of the volume of $H_3PO_4$ to be added had to be tried out experimentally. On the one hand a too strong acidification will not allow of an initial development and on the other hand in a weakly acid solution the alkaline substances resulting from the metabolic processes of the fungus will prevent the occurrence of a final very low pH. A suitable volume having been ascertained and the final pH not surpassing about 2.5, no sporangia developed (Fig. 2). Again when such an acid solution had been provided with some Mn, sporangia developed. The optimal development of the sporangia occurred more or less in tufts like in the medium with the ammonium salt. Here as well acid conditions appeared to be indispensable for the suppression of sporangia in the absence of manganese.

Fig. 2. $KNO_3$ as source of nitrogen.
flask 1: — Mn + $H_3PO_4$ (2.5 ml 3.21 n)
flask 2: — Mn — $H_3PO_4$

Once the method established for preparing cultures free from Mn which would show clear-cut deficiency symptoms, the way was open for the investigation into the possibility of composing a graded scale for manganese.

*a*). Nitrate as a source of nitrogen.

The initial experiment was carried out with $KNO_3$ as source of nitrogen, 2.5 ml $H_3PO_4$ being added to the individual cultures. In Table I Exp. 1 and 2 the results are presented.

Table I. Medium with $KNO_3$. Exp. 1 and 2 with glucose, Exp. 3, 4*a* and 5*a* with sucrose, Exp. 4*b* and 5*b* with sucrose and recrystallized $KNO_3$.

| Exp. | Per culture | | pH | | Sporangia | Yield in g | Yield as % of (— Mn) cultures |
|---|---|---|---|---|---|---|---|
| | ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| **Acidified medium** | | | | | | | |
| 1 | 2.5 | 0 | 1.6 | 1.6 | — | 0.765 | 100 |
| | 2.5 | 0.1 | 1.6 | 2.3 | + | 0.720 | 94 |
| | 2.5 | 0.2 | 1.6 | 2.5 | +++ | 0.640 | 84 |
| | 2.5 | 0.4 | 1.6 | 2.4 | +++± | 0.655 | 86 |
| | 2.5 | 0.8 | 1.6 | 2.5 | ++++ | 0.670 | 88 |
| 2 | 2.5 | 0 | 1.6 | 2.2 | — | 0.863 | 100 |
| | 2.5 | 0.05 | 1.6 | 2.2 | ± | 0.857 | 99 |
| | 2.5 | 0.1 | 1.6 | 2.2 | + | 0.675 | 78 |
| | 2.5 | 0.2 | 1.6 | 2.3 | +++ | 0.676 | 78 |
| | 2.5 | 1.0 | 1.6 | 2.3 | ++++ | 0.642 | 74 |
| 3 | 2.5 | 0 | 1.6 | 2.5 | — | 0.85 | 100 |
| | 2.5 | 0.005 | 1.6 | 2.5 | — | 0.83 | 98 |
| | 2.5 | 0.01 | 1.6 | 2.5 | — | 0.82 | 97 |
| 4*a* | 2.5 | 0 | 1.6 | 2.2 | — | 0.83 | 100 |
| | 2.5 | 0.005 | 1.6 | 2.2 | — | 0.75 | 90 |
| | 2.5 | 0.01 | 1.6 | 2.2 | — | 0.72 | 87 |
| 4*b* | 2.5 | 0 | 1.6 | 2.2 | — | 0.82 | 100 |
| | 2.5 | 0.005 | 1.6 | 2.2 | — | 0.77 | 94 |
| | 2.5 | 0.01 | 1.6 | 2.2 | — | 0.85 | 104 |
| **Non-acidified medium** | | | | | | | |
| 5*a* | 0 | 0 | 6.4 | 5.2 | +++ | 0.79 | 100 |
| | 0 | 0.005 | 6.4 | 5.2 | +++± | 0.885 | 112 |
| | 0 | 1 | 6.4 | 5.2 | ++++ | 0.915 | 116 |
| 5*b* | 0 | 0 | 6.4 | 5.2 | +++± | 0.755 | 100 |
| | 0 | 0.005 | 6.4 | 5.2 | +++± | 0.795 | 105 |
| | 0 | 1 | 6.4 | 5.2 | ++++ | 0.787 | 104 |

— = complete suppression of sporangia; ++++ = optimal development.

In the absence of any intentionally added manganese sporangia are completely lacking; a minute dose of 0.05 $\gamma$ induces a slight growth of sporangia, 0.1 $\gamma$ allows of a slightly denser growth, whilst a culture with 0.4 $\gamma$ is hardly to be distinguished from optimal development. These differences, however, covering merely such a small range, could not serve the purpose of a manganese standard.

In Exp. 3–5 sucrose has been supplied as carbohydrate, glucose of sufficient purity being no longer available. The sucrose has been purified by crystallization out of methylalcohol. STEINBERG

who in his experiments generally supplied sucrose, could not note any difference in effect between both sugars (27). In my experiments sucrose induced a somewhat slower growth so that the differences in sporangial development were later to occur than in the presence of glucose.

In Exp. 3–5 a range of smaller differences between the initial degrees was put up. In the unacidified medium (Exp. 5) hardly any difference existed between the production of sporangia in the (— Mn) cultures and those supplied with Mn.

Column 7 and 8 of Table I show that when the yields in weight are compared in the acidified media increase in the supply of Mn does not correlate with an increase in yield, on the contrary the (— Mn) cultures, where development of sporangia is completely suppressed, are highest in yield. This phenomenon will be discussed more in detail later.

In the non-acidified series (Exp. 5) where the (— Mn) cultures had developed sporangia, the addition of 0.005 $\gamma$ Mn results in a slightly higher yield. Again the sensitiveness for traces of Mn is such and differences noted are so slight that a weighing of the fungus mats would not supply any information as to their provision with manganese.

In Exp. 4 and 5 the effect on the yield between a medium provided with $KNO_3$ Merck pro analyse and one with recrystallized pro analyse can be compared. In the unacidified medium the sporangial growth was profuse, the yield, however, in the medium provided with the recrystallized salt lower than in the other. It might be conceived that the purification of the solution had been such that a deficiency of some other oligopleront had occurred. This, however, has not been further investigated. The acidified medium furnished no lower yield and its sensitiveness for traces of Mn was the same as in case of the usual brand. The $KNO_3$ Merck pro analyse may be deemed free from any contamination with Mn.

When the results of STEINBERG (23–27) are compared with the above a marked difference may be noted. The yield of his — non-acidified — cultures remains always significantly lower in the absence of Mn than in its presence. He uses, however, $NaNO_3$ in stead of $KNO_3$ as a source of nitrogen. So I put up a range with $NaNO_3$ in order to ascertain whether this might explain the discrepancy. In Table II the results are registrated.

In Exp. 1 on the non-acidified medium the (— Mn) solution gives rise to a fair amount of sporangia and a dose as low as 0.1 $\gamma$ Mn induces their optimal development. The weight of the yield in the absence of Mn equals that of the others. In Exp. 2 the effect of smaller doses of Mn is compared. The yield of the (— Mn) cultures is distinctly higher than in the presence of 0.01 $\gamma$ Mn and the sporangial development is slighter. When Exp. 2 was repeated with sucrose as source of carbohydrate, similar results were arrived at.

Table II. Medium with $NaNO_3$ and glucose.

| Exp. | Per culture | | pH | | Sporangia | Yield in g | Yield as % of (— Mn) cultures |
|---|---|---|---|---|---|---|---|
| | ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| | | | Non-acidified medium | | | | |
| 1 | 0 | 0 | 6.6 | 5.2 | ++ | 0.662 | 100 |
| | 0 | 0.005 | 6.6 | 5.2 | +++ | 0.672 | 102 |
| | 0 | 0.01 *) | 6.6 | 5.2 | ++++ | 0.695 | 105 |
| | 0 | 0.2 *) | 6.6 | 5.2 | ++++ | 0.665 | 101 |
| 2 | 0 | 0 | 6.6 | 5.5 | + | 0.77 | 100 |
| | 0 | 0.005 | 6.6 | 5.5 | +± | 0.71 | 92 |
| | 0 | 0.01 | 6.6 | 5.2 | ++ | 0.70 | 91 |
| | 0 | 0.025 | 6.6 | 5.2 | ++ | 0.75 | 97 |
| | | | Acidified medium | | | | |
| 3 | 2.5 | 0 | 1.6 | 1.6 | — | 0.256 | 100 |
| | 2.5 | 0.05 | 1.6 | 3.6 | + | 0.68 | 266 |
| | 2.5 | 0.1 | 1.6 | 3.6 | + | 0.77 | 301 |
| | 2.5 | 0.2 | 1.6 | 3.0 | ++ | 0.74 | 289 |
| | 2.5 | 0.3 | 1.6 | 2.8 | +++ | 0.76 | 297 |
| | 2.5 | 0.4 | 1.6 | 2.5 | +++± | 0.75 | 293 |

*) not in duplo.


Fig. 3. $NaNO_3$ as source of nitrogen.
flask 1: — Mn + $H_3PO_4$ (2.5 ml 3.21 n)
flask 2: 0.05$\gamma$ Mn + $H_3PO_4$ (2.5 ml 3.21 n)

When acidified by 2.5 ml $H_3PO_4$ the final pH attained by the medium was 1.6, and 0.05 $\gamma$ Mn induced a distinct development of sporangia (Fig. 3).

A gift of 0.4 $\gamma$ resulted in the nearly optimal development. The yield of mycelium in the absence of Mn was very materially lower than in its presence; 0.1 $\gamma$ Mn brought the yield on the level of higher gifts.

In another experiment the effect on visual growth of increasing amounts of $H_3PO_4$ in the absence of Mn is compared. 1.5 ml induced a final pH of 3.2, 2 ml of 2.5 and 2.5 ml of 2.2. Fig. 4 shows clearly the difference in development of sporangia; pH 3.2 allows of an optimal development, whilst 2.2 inhibits the growth of sporangia completely. So on this medium *Aspergillus niger* is very sensitive for slight differences in pH.

Fig. 4. $NaNO_3$ as source of nitrogen.
flask 1: — Mn + 1.5 ml $H_3PO_4$
flask 2: — Mn + 2.0 ml $H_3PO_4$
flask 3: — Mn + 2.5 ml $H_3PO_4$

In order to study the influence of lower amounts of Mn on the yield, Exp. 3 of Table II was repeated. Pure glucose being no longer available it has been substituted by sucrose.

Table III. Medium $NaNO_3$ and sucrose.

| Exp. | Per culture | | pH | | Spo-rangia | Yield in g | Yield as % of (— Mn) cultures |
|---|---|---|---|---|---|---|---|
| | ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| 1 | 2.5 | 0 | 1.6 | 1.6 | — | 0.115 | 100 |
| | 2.5 | 0.005 | 1.6 | 1.6 | — | 0.34 | 296 |
| | 2.5 | 1 | 1.6 | 1.6 | + | 0.774 | 673 |
| | 2 | 0 | 1.6 | 1.6 | — | 0.315 | 100 |
| | 2 | 0.005 | 1.6 | 1.6 | — | 0.46 | 146 |
| | 2 | 1 | 1.6 | 1.6 | +++ | 0.80 | 254 |
| 2 | 2.5 | 0 | 1.6 | 1.6 | — | 0.44 | 100 |
| | 2.5 | 0.005 | 1.6 | 1.6 | — | 0.625 | 142 |
| | 2.5 | 0.01 | 1.6 | 1.6 | — | 0.57 | 130 |
| | 2 | 0 | 1.6 | 2.3 | — | 0.772 | 100 |
| | 2 | 0.005 | 1.6 | 2.3 | — | 0.765 | 99 |
| | 2 | 0.01 | 1.6 | 2.3 | — | 0.760 | 98 |
| | 2 | 0.1 | 1.6 | 2.3 | — | 0.775 | 100 |
| 3 | 2.5 | 0 | 1.6 | 2.2 | — | 0.76 | 100 |
| | 2.5 | 0.005 | 1.6 | 2.2 | — | 0.83 | 109 |
| | 2.5 | 0.01 | 1.6 | 2.2 | — | 0.83 | 109 |
| | 2 | 0 | 1.6 | 3.5 | — | 0.89 | 100 |
| | 2 | 0.005 | 1.6 | 3.5 | — | 0.87 | 98 |
| | 2 | 0.01 | 1.6 | 3.5 | — | 0.87 | 98 |

Table III shows that as long as the final pH remains very low, the marked increase in weight induced by a dose as low as 0.005 $\gamma$ Mn may be noted. When, however, the final pH is somewhat higher (Exp. 3) this influence is much slighter. When 2 ml $H_3PO_4$ has been supplied the yield of the (— Mn) cultures equalled that of the others.

*b*). Ammonium nitrate as a source of nitrogen.

As a difficulty met with in both former media lay in the fact that they had to be acidified strongly in order to maintain a high H-ion concentration up till the end, this involving the risk of inhibition of the initial development, $NH_4NO_3$ was tried as a source of nitrogen. It was indeed to be expected that the trend for alkalinisation would be weaker in such a medium. In fact when 3.1 g $NH_4NO_3$ per liter had been supplied to the medium, 1 ml $H_3PO_4$ appeared sufficient for keeping the pH below 1.9.

Table IV shows that 0.8 $\gamma$ Mn is needed for a development of sporangia very near the optimal. So the acidified $NH_4NO_3$ medium is less sensitive in its response for minute traces of Mn than both the former solutions and a larger number of visually to be distinguished degrees can be realised. As far as this goes it might prove more suitable for practical purposes. Fig. 5 represents such a graded scale.

Table IV. Acidified medium with $NH_4NO_3$ and glucose.

| Per culture | | pH | | Sporangia | Yield in g | Yield as % of (— Mn) cultures |
|---|---|---|---|---|---|---|
| ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| 1 | 0 | 1.6 | 1.9 | — | 0.585 | 100 |
| 1 | 0.05 | 1.6 | 1.6 | ± | 0.58 | 99 |
| 1 | 0.1 | 1.6 | 1.9 | ± | | |
| 1 | 0.2 | 1.6 | 1.9 | +± | | |
| 1 | 0.4 | 1.6 | 1.9 | +++ | | |
| 1 | 0.6 | 1.6 | 1.9 | +++ | | |
| 1 | 0.8 | 1.6 | 1.9 | +++± | | |
| 1 | 1 | 1.6 | 1.9 | ++++ | 0.64 | 109 |

In order to study the effect on the yield in weight of small doses of Mn in an acidified medium the experiment of Table V was put up.

Fig. 5. $NH_4NO_3$ as source of nitrogen; 1 ml $H_3PO_4$ added. flask 1: — Mn; flask 2: + 0.1 $\gamma$ Mn; flask 3: + 0.2 $\gamma$ Mn; flask 4: + 0.5 $\gamma$ Mn.

Table V. Acidified medium with $NH_4NO_3$ and sucrose.

| Per culture | | pH | | Sporangia | Yield in g | Yield as % of (— Mn) cultures |
|---|---|---|---|---|---|---|
| ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| 1.5 | 0 | 1.6 | 1.6 | — | 0.97 | 100 |
| 1.5 | 0.005 | 1.6 | 1.6 | · | 0.765 | 79 |
| 1.5 | 0.01 | 1.6 | 1.6 | — | 0.885 | 91 |

In contrast with the results noted in the $NaNO_3$ medium, here the omission of Mn in a medium with a high final acidity does not result in a low yield. Apparently it is not the acidity of the medium as such which involves a weak growth of mycelium in the absence of Mn.

Table VI. Medium with $NH_4NO_3$ and glucose.

| Per culture | | pH | | Sporangia | Yield in g | Yield as % of 1 ml $H_3PO_4$ culture |
|---|---|---|---|---|---|---|
| ml $H_3PO_4$ | $\gamma$ Mn | initial | final | | | |
| 1 | 0 | 1.6 | 1.6 | — | 0.585 | 100 |
| 0.5 | 0 | 1.9 | 2.5 | ++++ | 0.59 | 101 |
| 0 | 0 | 6.7 | 3.8 | ++++ | 0.50 | 85 |
| 1 | 0.05 | 1.6 | 1.6 | ± | 0.58 | 100 |
| 0.5 | 0.05 | 1.9 | 2.5 | ++++ | 0.59 | 102 |
| 0 | 0.05 | 6.7 | 3.8 | ++++ | 0.51 | 88 |

Table VI learns that the medium with $NH_4NO_3$ as well is very sensitive for slight raisings of pH. As may be taken from Table VI the addition of 0.5 ml $H_3PO_4$ will result in a final pH of 2.5; under these conditions the (— Mn) cultures will produce optimal growth of sporangia, the pH rendering the presence of Mn superfluous.

c). Ammonium as a source of nitrogen.

Finally the action of ammonium as only source of nitrogen was tested. Most of these experiments have been carried out with $NH_4Cl$, a lesser number with $(NH_4)_2SO_4$; the results arrived at were of a same nature.

When no Mn has been intentionally added a coalescent white felt is formed and any development of sporangia is suppressed. The acidification occurs at a quick rate and the final pH is 1.6. The addition of small doses of Mn induces the growth of sporangia; their development, however, is always less dense than when nitrate has been supplied. In a fairly wide range (0.1 $\gamma$ up till 0.7 $\gamma$, Fig. 6) the development may remain sparse and no clear-cut differences may be met with. And even the optimal development will not produce a completely covered black mat: the sporangia will occur in evenly scattered black tufts with small uncovered regions between. This has to be ascribed to the acidity of the medium involved by the development of the fungus. In the acidified nitrate medium a similar phenomenon has been stated to occur.

The amounts of manganese inducing equal degrees of sporangial development varied considerably in the various experimental sets, duplo's however corresponding closely. Fig. 6 in which the curves connect the amounts of manganese inducing an equal sporangial development in various sets, illustrates this clearly. It might be conceived that this erratic behaviour was caused by

variety in amounts of the inoculum, the suspensions used being of varying density. It was attempted to answer this experimentally. In media containing 0.3 $\gamma$ and 0.5 $\gamma$ Mn per culture inocula consisting out of 1, 3 or 5 drops did not, however, influence the resulting degree of development. Thus the variety in the density of sporangia in equal concentrations of manganese may not be ascribed to a difference in the amount of spores in the inoculum. Still a difference in rate or measure of acidification of the medium will probably be cause of the variety. As long as this discrepancy in results may not be prevented, an unbuffered medium with ammonium as only source of nitrogen is quite unsuitable as a visual manganese standard.

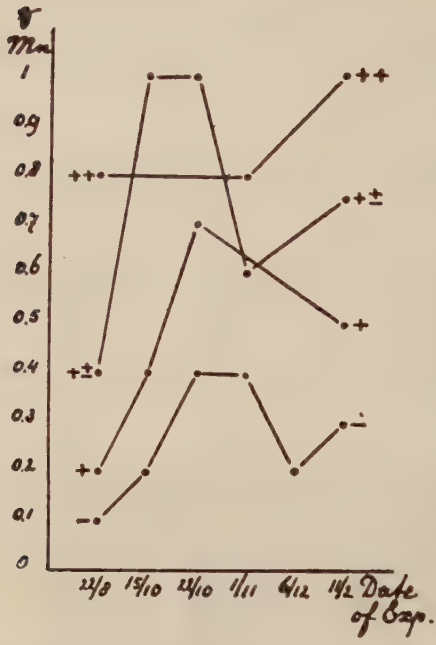


Fig. 6.
Amounts of manganese inducing equal sporangial development in various experimental sets.

The weight of the yields induced by the various amounts of Mn has been ascertained. Table VII offers an example.

Table VII. Medium with $NH_4Cl$ and glucose.

| Per culture | pH | | Sporangia | Yield in g | Yield as % of (— Mn) culture |
|---|---|---|---|---|---|
| $\gamma$ Mn | initial | final | | | |
| 0 | 6.6 | 1.9 | — | 0.564 | 100 |
| 0.1 | 6.6 | 1.9 | — | 0.51 | 90 |
| 0.25 | 6.6 | 1.9 | — (±) | 0.59 | 105 |
| 0.5 | 6.6 | 1.9 | +± | 0.61 | 108 |
| 0.75 | 6.6 | 1.9 | ++ | 0.555 | 98 |
| 1 | 6.6 | 1.9 | ++ | 0.58 | 103 |
| 1.25 | 6.6 | 1.9 | ++ | 0.595 | 106 |
| 1.5 | 6.6 | 1.9 | ++ | 0.545 | 97 |

There is no definite trend in weight of the yield induced by increasing amounts of manganese. When the yield for 0.1 $\gamma$ Mn is expressed as % of the yield in the absence of Mn, four different experimental sets offer as results 105%, 99%, 95% and 90%. So the yield in the absence of Mn may easily be higher than in its presence and an ascertainment by weight of the yields will not allow of any conclusions as to their content of Mn.

The effect was tried of a supply of $CaCO_3$ to the medium as a buffer, which as has been stated already, will allow of a profuse development of sporangia in the absence of manganese. For this purpose rigidly pure $CaCO_3$ had been prepared. The optimal dose of $CaCO_3$ per culture had to be ascertained. Table VIII presents the results.

Table VIII. Medium with $NH_4Cl$ and glucose.

| Exp. | Per culture | | pH | | Spo-rangia | Yield in g | Yield as % of | |
|---|---|---|---|---|---|---|---|---|
| | g $CaCO_3$ | $\gamma$ Mn | initial | final | | | (–$CaCO_3$) culture | (— Mn) culture |
| 1 | 0 | 0 | 6.6 | 1.6 | — | 0.6 | 100 | |
| | 0.062 | 0 | 6.6 | 1.9 | +± | 0.72 | 120 | |
| | 0.125 | 0 | 6.6 | 3.8 | ++ | 1.15 | 192 | |
| | 0.250 | 10 | 6.6 | 6.1 | ++ | 0.94 | 157 | |
| | 0 | 10 | 6.6 | 1.6 | +++ | 0.515 | 100 | |
| | 0.062 | 10 | 6.6 | 2.1 | +++ | 0.6 | 117 | |
| | 0.125 | 10 | 6.6 | 4.1 | ++++ | 1.225 | 238 | |
| | 0.250 | 10 | 6.6 | 6.1 | ++++ | 0.945 | 183 | |
| 2 | 0.125 | 0 | 6.6 | 5.5 | +++± | 0.740 | | 100 |
| | 0.125 | 0.05 | 6.6 | 6.1 | ++++ | 0.785 | | 106 |
| | 0.125 | 0.10 | 6.6 | 5.5 | ++++ | 0.780 | | 105 |
| | 0.125 | 0.20 | 6.6 | 5.5 | ++++ | 0.780 | | 105 |

It shows clearly that in the absence as well as in the presence of Mn a dose of 0.125 g $CaCO_3$ produced the highest yields, respectively 192% and 238% of the amount in the absence of $CaCO_3$. A dose of 0.25 g is apparently too high; the final pH of 6.1 will presumably induce less favourable growing conditions for *Aspergillus niger*, be it that some nutritional substance has been precipitated. In the absence of Mn the cultures with a final pH of 1.6 and 2.1 have produced a higher yield in weight than the corresponding cultures which had been supplied with 1.0 $\gamma$ Mn. The latter cultures had developed sporangia more profusely which may have influenced unfavourably the amount of mycelium synthetised. The yield of the cultures with the larger amounts of $CaCO_3$ was markedly higher than any yields arrived at in the nitrate series.

The experiments with an ammonium salt as only source of nitrogen indicate, that the ascertainment by weight of the yields for increasing amounts of Mn cannot furnish a manganese standard.

## 3. Discussion.

The experiments have furnished evidence that in a medium of very low pH manganese is essential for the development of sporangia. Is it, however, essential as well for the development of mycelium?

Table IX.

| Culture medium | — Mn | | With highest sporangia development | | | | | With 0.005 γ Mn | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Final pH | Spor. | Final pH | Spor. | γ Mn | Yield in g | Yield as % of (— Mn) culture | Final pH | Spor. | Yield in g | Yield as % of (— Mn) culture |
| $KNO_3$, glucose | 1.6 | — | 2.5 | ++++ | 0.8 | 0.670 | 88 | | — | | |
| idem | 2.2 | — | 2.3 | ++++ | 1.0 | 0.642 | 74 | | — | | |
| $KNO_3$, sucrose | 2.2 | — | | | | | | 2.5 | | 0.83 | 98 |
| $KNO_3$ recryst. sucrose | 2.5 | — | | | | | | 2.2 | | 0.77 | 94 |
| $NaNO_3$, glucose | 1.6 | — | 2.5 | +++± | 0.4 | 0.75 | 293 | | | | |
| $NaNO_3$, sucrose | 1.6 | — | 1.6 | + | 1.0 | 0.774 | 673 | 1.6 | — | 0.34 | 296 |
| idem | 1.6 | — | 1.6 | +++ | 1.0 | 0.80 | 254 | 1.6 | — | 0.46 | 146 |
| idem | 1.6 | — | | | | | | 1.6 | — | 0.625 | 142 |
| idem | 2.3 | — | | | | | | 2.3 | — | 0.765 | 99 |
| idem | 2.2 | — | | | | | | 2.2 | — | 0.83 | 109 |
| idem | 3.5 | — | | | | | | 3.5 | — | 0.87 | 98 |
| $NH_4NO_3$, glucose | 1.9 | — | 1.9 | +++± | 1 | 0.64 | 109 | | | | |
| idem | 1.6 | — | 1.6 | ± | 0.05 | 0.58 | 99 | | | | |
| $NH_4NO_3$, sucrose | 1.6 | — | | | | | | 1.6 | — | 0.765 | 79 |

In Table IX have been brought together the data bearing on this question of the experimental sets in which a low pH has involved the suppression of sporangia in the absence of Mn. The yields of the (— Mn) cultures are compared with those of cultures which had attained the best sporangia development in the particular set and (or) with the yields of 0.005 $\gamma$ Mn cultures. In the $KNO_2$ sets the yields of the (— Mn) cultures without sporangia are higher than those of cultures where the supply of manganese had involved development of sporangia. It may be conceived that as a result of the formation of sporangia some nutritional substance other than Mn might come in the minimum; this would involve an inhibition of the growth of mycelium at an earlier stage than in the absence of manganese. On the $NH_4NO_3$ medium no definite trend in the relation of the yields in absence or presence of Mn could be ascertained, neither could this be stated for $NH_4Cl$.

On the medium with $NaNO_3$, however, the yield in the absence of Mn is very markedly lower than in its presence. As long as a very low pH prevails under strongly acid conditions manganese is here apparently essential for the development of mycélia as well as of sporangia. Like acid conditions, however, do not involve a similar inhibition of mycelial development when other sources of nitrogen have been supplied to the medium. Thus the marked feature of $NaNO_3$ cultures may not be wholly ascribed to the influence of a low pH, although it is a condition *sine qua non* for its occurrence. As has been stated already the deviating results with the $NaNO_3$ medium apparently neither may be ascribed to a greater freedom from last traces of manganese of this salt. So the discrepancy has to be ascribed to a difference in metabolism on the acidified $NaNO_3$ medium.

Table X contains data of less acid sets where sporangia developed in the absence of manganese. Under those conditions no definite trend in the relation between mycelial yield in the absence and presence of Mn can be ascertained and under these conditions $NaNO_3$ induces no deviation in behaviour.

Might it, however, be possible to explain the occurrence of deficiency symptoms in either the sporangial or the mycelial development in highly acid media by an action of the medium itself on the availability of manganese? In a more acid medium manganese might on the contrary be expected to exist in a more soluble form and thus manganese deficiency would be less apt to occur. Thus the case of the difference in action between media of lower and higher pH has in fact not to be looked for in an action in the medium but in an action of the fungus.

So the intake of manganese by *Aspergillus niger* under varying conditions has been studied by means of estimation of the content of Mn in the yield. In this end the culture media had been supplied with higher gifts of Mn, 150 $\gamma$ or 200 $\gamma$ per culture. The fungous mats were removed out of the culture flasks, rinsed in water, dried at 105° C. and weighed. The amount of manganese was

Table X.

| Culture medium | — Mn | | With highest sporangial development | | | | |
|---|---|---|---|---|---|---|---|
| | Final pH | Spor. | Final pH | Spor. | γ Mn | Yield in g | Yield as % of (— Mn) culture |
| $KNO_3$, sucrose | 5.2 | ++++ | 5.2 | ++++ | 1 | 0.915 | 116 |
| $KNO_3$ recryst. sucrose | 5.2 | ++++ | 5.2 | ++++ | 1 | 0.787 | 104 |
| $NaNO_3$, glucose | 5.2 | ++ | 5.2 | +++ | 0.01 | 0.695 | 105 |
| idem | 5.5 | ± | 5.2 | ++ | 0.01 | 0.700 | 91 |
| $NH_4NO_3$, glucose | 2.5 | ++++ | 2.5 | ++++ | 0.05 | 0.590 | 101 |
| idem | 3.8 | ++++ | 3.8 | ++++ | 0.05 | 0.510 | 88 |
| $NH_4Cl$, glucose | 3.8 | ++ | 4.1 | ++++ | 10 | 1.225 | 110 |
| idem | 6.1 | +++± | 6.1 | ++++ | 0.05 | 0.785 | 106 |

Table XI.

| Medium | γ Mn | Number of cultures | pH | | Yield in g per culture | γ Mn in 0.5 g |
|---|---|---|---|---|---|---|
| | | | initial | final | | |
| $NH_4Cl$ + 0.25 g $CaCO_3$ | 200 | 1 | 6.6 | 5.8 | 0.83 | 30 |
| idem | 200 | 1 | 6.6 | 5.8 | 0.79 | 20 |
| $NH_4Cl$ | 200 | 1 | 6.6 | 1.6 | 0.56 | 15 |
| idem | 200 | 1 | 6.6 | 1.6 | 0.57 | 20 |
| $NH_4NO_3$ | 200 | 1 | 6.4 | 3.2 | 0.73 | 22.5 |
| idem | 200 | 1 | 6.4 | 2.9 | 0.76 | 40 |
| idem | 200 | 1 | 6.4 | 3.7 | 0.66 | 22.5 |
| idem | 200 | 3 | 6.4 | 3.4 | 0.62 | 40 |
| $NH_4NO_3$ + 1 ml $H_3PO_4$ | 200 | 1 | 1.6 | 1.9 | 0.66 | 15 |
| idem | 200 | 1 | 1.6 | 1.9 | 0.69 | 17.5 |
| idem | 200 | 1 | 1.6 | 1.9 | 0.65 | 15 |
| idem | 200 | 3 | 1.6 | 1.6 | 0.61 | 5 |
| $KNO_3$ | 200 | 3 | 6.4 | 5.2 | 0.74 | 30 |
| $KNO_3$ + 2 ml $H_3PO_4$ | 200 | 3 | 1.6 | 2.8 | 0.69 | 10 |
| $NaNO_3$ | 150 | 3 | 6.4 | 6.7 | 0.67 | 20 |
| $NaNO_3$ + 2 ml $H_3PO_4$ | 150 | 3 | 1.6 | 2.8 | 0.77 | 10 |

estimated according to the method of MARSHALL (12), amended by STEENBJERG (22).

A sample of 0.5 g dry weight is transferred to a Kjeldahl flask, 5 ml $HNO_3$ and 6 ml $H_2SO_4$ are added and the flask is heated on a low flame, later on a higher until the liquid is colourless. After addition of 15 ml distilled water the solution is neutralized with 1 ml 6 n NaOH, and 1.25 ml $H_2SO_4$ is added in excess. The solution is filtered of and the filter washed with boiling water. 1 ml 6 n $HNO_3$ and 2 ml 0.1 n $AgNO_3$ are added. After the solution has been supplied with a spatula ammonium persulphate it is carefully heated (on the verge of boiling) up till the appearance of the pink shade of permanganate. After one more minute near boiling temperature it is cooled down and transferred to a colorimeter glass of 100 ml. The colorimeter scale ranges from 5—65 $\gamma$ Mn and is sensitive for differences of 5 $\gamma$. In order to render the standard solution more permanent it is provided with some potassium perjodate.

Table XI presents the results. The relatively high dose of 200 $\gamma$ Mn per culture induced no high intake, 40 $\gamma$ Mn/0.5 g being the maximum. Differences in intake are slight. The strongly acid cultures show a somewhat lower content of the yield than the cultures with a higher final pH. So it is not quite out of question, that the fact that sporangia will develop in media of lesser acidity in the absence of intentionally added manganese, might be caused by a better intake under those conditions of still prevailing traces of manganese. As such traces cannot be detected chemically, direct evidence cannot be gained.

As has been stated already the sporangial development in an unbuffered ammonium salt medium responds less quickly on traces of Mn than in a nitrate medium. The data in table XI prove that this may not be explained by a weaker intake of Mn in the former medium. The difference must be due to a difference between the metabolic processes of the fungus in both media.

It was further tested whether the intake of manganese would correspond with an increase in supply of the medium. As such a buffered $NH_4Cl$ medium was used.

Table XII.

| $\gamma$ Mn | Number of cultures | pH | | Yield in g per culture | $\gamma$ Mn in 0.5 g |
|---|---|---|---|---|---|
| | | initial | final | | |
| 10 | 2 | 6.6 | 5.8 | 0.685 | 5 |
| 20 | 2 | 6.6 | 5.8 | 0.825 | 5 |
| 40 | 1 | 6.6 | 5.8 | 0.750 | 10 |
| 40 | 1 | 6.6 | 6.1 | 0.580 | 15 |
| 80 | 1 | 6.6 | 6.4 | 0.740 | 15 |
| 80 | 1 | 6.6 | 6.1 | 0.740 | 10 |

Table XII shows that no such correlation exists. Estimation of the content of manganese of the yield in mycelium would not

provide any information about the amount of manganese supplied to the fungus,

The results arrived at indicate, that manganese plays quite another part in the metabolism of *Aspergillus niger* than molybdenum, although for oats results of BURSTRÖM and LUNDEGAARDH might seem to point at some similar action. Whilst molybdenum is merely essential when nitrate is the only source of nitrogen, in case of manganese the difference between nitrate and ammonium plays no part. The chief deficiency symptom, the suppression of sporangia, however, depends on the pH of the medium and only occurs at a low pH. At a higher pH sporangia will develop undependently of the prevailing source of nitrogen. Under such conditions all essential synthetic processes are apparently possible in the absence of manganese. Merely a deeper biochemical investigation of the metabolism of *Aspergillus niger* might furnish some explanation for the difference in need of manganese under various conditions.

My results need comparison with those arrived at by other investigators.

BERTRAND was able to induce differences in yield by the supply of 0.005 $\gamma$ Mn when compared with (— Mn) cultures. When $NaNO_3$ had been supplied as source of nitrogen I arrived at the same result. Apparently the simple method of purification by adsorption served as well for the removal of the last traces of manganese as the more elaborate means applied by BERTRAND.

Between the results of STEINBERG (25. 26) and mine some discrepancies are manifest, although I have meant to follow his methods exactly. Without any previous acidification of the culture media the final pH of his various nitrate solutions ranges from 1.58—2.11 and is thus much lower than I could ascertain in corresponding experiments. This may explain, however, that in the not intentionally acidified nitrate media STEINBERG often notes a complete suppression of sporangia in the absence of manganese. In some experiments, however, sporangia did occur; for instance a culture with $NaNO_3$ and a final pH of 1.89 and another with $NH_4NO_3$ and a final pH of 1.67 produced sporangia at about the level designed by me as +. Moreover he could note in the absence of Mn a constantly smaller yield of mycelium in its presence: f.i. a culture with $NH_4NO_3$ produced 68% of the yield of one supplied with Mn. In my experiments I could merely note in a highly acidified $NaNO_3$ medium a lower yield of mycelium in the absence of Mn than in its presence. I am unable to indicate the cause of these discrepancies.

In a paper of BORTELS in 1927 (6) on the significance of iron, zinc and copper for *Aspergillus niger*, he notes that in acid media growth is slight and formation of spores is suppressed; when such a culture solution is rendered more alkaline sporangia will develop abundantly. He was the first to introduce the purification of the medium by adsorption on coal and did not intentionally supply

any manganese. Although he looks for an explanation in a direct action of H′ in the presence of much Zn, it seems to me very probable that a manganese deficiency had come into play there.

Finally a graded scale for manganese has to be considered as to its suitability for a practical soil test. When merely the response to increasing amounts of Mn is kept in view, the acidified scale with $NH_4NO_3$ as source of nitrogen might seem promising. For carrying out a soil test a soil sample of known weight has to be transferred to the culture medium. In any alkaline soil, however, some insoluble manganese may be expected to occur, which at the low pH of the medium will be converted into a manganous salt and thus will go into solution. Any manganese detected by means of the development of sporangia would prove nothing about the actual occurrence of plant available manganese in the soil sample. Alkaline soils, thus soils in need of estimation of their available manganese, might only be tested by means of an about neutral buffered solution. In such a medium, however, manganese appears to be unessential for the development of sporangia.

Neither would an estimation of the content of Mn in the yield of mycelium furnish any information about the amount supplied.

For practical purpose as a test for plant available manganese the *Aspergillus* method, such I could contrive to make it up, appears to be unsuitable.

## Summary.

1. The response of *Aspergillus niger* to the presence of manganese in the nutrient solution has been ascertained.
2. In an initially acidified medium which kept up its acidity or in an unbuffered neutral medium which grew strongly acid, manganese appeared essential for sporangial development.
3. In suitably acidified solutions $KNO_3$ as source of nitrogen induced slight sporangial development in the presence of 0.05 $\gamma$ Mn, $NaNO_3$ of 0.025 $\gamma$ Mn and $NH_4NO_3$ of 0.05 $\gamma$ Mn. In the acidified $NH_4NO_3$ medium the greatest number of visually to be distinguished degrees could be ascertained.
4. In acidified solutions with $KNO_3$ the yield of mycelium was higher in the absence of manganese than in cultures which in its presence had developed sporangia. Presumably the development of sporangia inhibits further development of mycelium.
5. The weight in yield of mycelium in an acidified $NaNO_3$ solution in the absence of manganese was very significantly lower than in the presence of 0.005 $\gamma$ Mn.
6. In non-acidified nitrate solutions sporangia developed in the absence of manganese. No definite trend in the yield of mycelium could be ascertained.
7. In unbuffered solutions with ammonium salts no sporangia developed in the absence of manganese. The response on

various amounts of manganese was erratic. No definite trend in mycelial development could be ascertained.

8. Solutions with an ammonium salt suitably buffered by $CaCO_3$ produced sporangia in the absence of manganese. They gave the highest yields in weight.
9. The intake of manganese by the fungus on various media offers slight differences. The intake is slightly higher in weakly acid than in strongly acid solutions.
10. The fact that manganese is only essential in a highly acid medium may not be ascribed to an action of the medium as such and must be caused by differences in the metabolism of *Aspergillus niger*.
11. As *Aspergillus niger* is sensitive for traces of manganese only under highly acid conditions an *Aspergillus* standard will not be suitable for soil tests.

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# RAPPORT ENTRE LA TENEUR EN HUMIDITÉ DU LAIT SEC MAIGRE (PROVENANT DE LAIT ÉCRÉMÉ) ET LE DÉVELOPPEMENT D'UNE FLORE MICROBIENNE DANS CE LAIT

par

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## 1. Introduction.

La bibliographie de la question nous apprend, que dans presque tous les laits secs, ou poudres de lait, le nombre de germes est bas et que pendant la conservation le nombre de micro-organismes augmente à peine ou point du tout, mais qu'au contraire il diminue le plus souvent. En général l'état de siccité de ces laits est considéré comme l'obstacle au développement d'une flore microbienne.

Les laits secs, surtout celui qui est obtenu par pulvérisation, possèdent une très grande hygroscopicité; dans un emballage non hermétique ils reprennent vite de l'humidité. Supplee (3) a établi l'équilibre des teneurs en humidité du lait sec en le conservant dans des milieux exposés à une tension de vapeur relative variant de 10 à 80%. Il a trouvé, le fait remarquable, que dans une humidité relative dépassant 40%, la reprise d'eau s'élevait en peu de temps à un maximum et qu'ensuite la teneur en humidité de la poudre rebaissait graduellement jusqu'à un certain point d'équilibre dépendant naturellement de l'humidité de l'air ambiant. Quand la teneur en humidité relative du milieu était au-dessus de 80%, la poudre commençait à moisir sous peu.

Supplee ne dit pas s'il a fait des recherches ultérieures sur la moisissure en rapport avec l'humidité de la poudre et l'air ambiant. Tamsma (4) a établi l'équilibre des teneurs en humidité d'un échantillon de lait sec sous tension de vapeur relative de 20 à 50%. Ses résultats sont essentiellement conformes à ceux de Supplee. Que Tamsma diffère environ de 1% avec Supplee dans les points d'équilibres des teneurs en humidité, cela importe peu, les poudres différant de composition par suite de leur fabrication et de leur provenance différentes.

Or, il était intéressant de chercher à suivre exactement quel est le rapport entre la teneur en humidité de la poudre et les possibilités d'existence des bactéries, des levures et des moisissures à la température du laboratoire.

Pour expérimenter la chose nous avons conservé du lait sec

à divers degrés d'humidité relative. Les différents milieux ont été constitués dans des exsiccateurs à l'aide de solutions saturées de sel. Dans ces exsiccateurs nous avons placé une cuvette de verre contenant le lait sec; nous avons pris comme objet d'expérience un échantillon de poudre Hatmaker[1]) et un de poudre Krause[2]) sous des tensions de vapeur relatives de 30%, 75%, 80%, 85%, 90% et de 100%.

Nous avons employé les solutions de sel indiquées dans l'aperçu suivant:

| | | | |
|---|---|---|---|
| Pour la tension de vapeur relative de | 30% | solution saturée de | $CaCl_2$ |
| „ „ „ „ „ „ „ | 75% | „ „ „ | NaCl |
| „ „ „ „ „ „ „ | 80% | „ „ „ | $(NH_4)_2SO_4$ |
| „ „ „ „ „ „ „ | 90% | „ „ „ | KCl |
| „ „ „ „ „ „ „ | 100% | eau | |

2. Conservation du lait sec écrémé (maigre) dans des milieux exposés à différentes tensions de vapeur relative.

Dans notre première série d'essais de conservation nous avons conservé la poudre en boîtes de Petri sans couvercle et avons gardé les exsiccateurs à la température du laboratoire sans les protéger contre la lumière du jour. A un moment donné nous avons pris un échantillon dans la couche supérieure et celui-ci a fait l'objet de recherches sur le nombre de germes et la teneur en humidité. La teneur en humidité a été déterminée par dessiccation, jusqu'à un poids constant, dans une étuve chauffée à 103° C., d'une quantité déterminée de poudre dans une cuvette de porcelaine. Le nombre total de germes a été déterminé sur des plaques d'autolysat de levure et de peptone. Après inoculation les plaques ont été incubés pendant quatre jours à 25° C.

Le résultat sous les différentes tensions de vapeur est mentionné dans les 2 tableaux suivants: le premier se rapporte à la poudre Krause et le deuxième à la poudre Hatmaker. Dans le cours de notre rapport nous parlons toujours, pour plus de simplicité, de lait sec (ou poudre) Hatmaker et de lait sec (ou poudre) Krause.

Résultats des recherches.

En examinant les résultats obtenus on remarque tout d'abord qu'il y a bien quelquefois des constatations qui paraissent un peu invraisemblables si on les compare avec l'ensemble.

Les fluctuations de la teneur en humidité trouvent probablement leur cause dans ce que, pour l'analyse, on a pris la couche supérieure et qu' après on y a mélangé la poudre restante. Il est

[1]) Le lait sec Hatmaker s'obtient en faisant passer le lait en lamelles entre deux cylindres tengents, chauffés à une certaine température. La dessiccation est instantanée.

[2]) Le lait sec Krause est obtenu en pulvérisant le lait en gouttelettes et en l'envoyant dans une chambre chauffée par un courant d'air sec et chaud.

Tableau I.

| durée de conservation en jours | humidité relative de: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100% | | 90% | | 85% | | 80% | | 75% | | 30% | |
| | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % |
| 0 | 15000 | 4.0 | 15000 | 4.0 | 11500 | 4.0 | 11500 | 4.0 | 18000 | 4.8 | 18000 | 4.8 |
| 4 | — | — | — | — | 12000 | — | 7000 | — | — | — | — | — |
| 8 | 2400 | 8.5 | 2000 | 9.4 | 8000 | 7.2 | 4000 | 6.7 | — | — | — | — |
| 13 | 1400 | 9.6 | 1300 | 10.6 | 2200 | — | 3000 | — | 1300 | — | 2500 | — |
| 17 | 1600 | 8.0 | 1500 | 8.4 | 1300 | 8.7 | 900 | 10.0 | — | — | — | — |
| 21 | 1100 | 10.2 | 1700 | 9.9 | 5000 | 9.8 | 1000 | 9.9 | — | — | — | — |
| 25 | 1300 | 9.9 | 1300 | 10.2 | 4000 | 9.4 | 4000 | 10.3 | — | — | — | — |
| 29 | 1000 | 10.3 | 1000 | 10.7 | 3000 | — | 1000 | — | 1400 | — | 2600 | — |
| 34 | 1100 | 13.1 | 1000 | 11.2 | 3000 | 9.5 | 1500 | 11.2 | — | — | — | — |
| 38 | 1000 | 13.9 | 1000 | 11.3 | 3000 | 10.5 | 1000 | 11.4 | — | — | — | — |
| 42 | 4000 | 14.1 | 3100 | 13.1 | 1500 | 10.9 | 1200 | 11.8 | — | — | — | — |
| 46 | 4000 | 13.0 | 3500 | 14.1 | 1 00 | 11.0 | 1500 | 11.9 | 1200 | — | 600 | — |
| 50 | 5700 | 16.5 | 5000 | 14.0 | — | — | — | — | — | — | — | — |
| 54 | 9500 | — | 21000 | — | — | 11.7 | 2500 | 12.0 | — | — | — | — |
| 58 | moisie | 18.4 | moisie | 15.9 | 1300 | 11.6 | 2100 | 11.8 | 700 | — | 300 | — |
| 63 | — | — | — | — | — | 12.6 | 2000 | 12.0 | — | — | — | — |
| 67 | — | — | — | — | 2000 | 12.1 | 2000 | 12.0 | — | — | — | — |
| 71 | — | — | — | — | 4700 | 12.3 | 15000 | 12.9 | 1700 | — | 600 | — |
| 75 | — | — | — | — | — | 12.4 | moisie | 12.9 | — | — | — | — |
| 83 | — | — | — | — | 3000 | 12.7 | — | — | — | — | — | — |
| 87 | — | — | — | — | 4300 | 14.2 | — | — | 1300 | — | 2800 | — |
| 91 | — | — | — | — | 1100 | 13.1 | — | — | — | — | — | — |
| 96 | — | — | — | — | 2300 | — | — | — | — | — | — | — |
| 100 | — | — | — | — | 15000 | 14.7 | — | — | 900 | — | 1700 | — |
| 104 | — | — | — | — | mo s e | 13.8 | — | — | — | — | — | — |
| 114 | — | — | — | — | — | — | — | — | 4500 | — | 7500 | — |
| 128 | — | — | — | — | — | — | — | — | 1300 | — | 900 | — |
| 142 | — | — | — | — | — | — | — | — | 400 | 10.8 | 1500 | 6.0 |
| 156 | — | — | — | — | — | — | — | — | 1600 | — | 500 | — |
| 180 | — | — | — | — | — | — | — | — | 1600 | 12.8 | — | — |

Tableau II.

| durée de conservation en jours | humidité relative de: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100% | | 90% | | 85% | | 80% | | 75% | | 30% | |
| | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % | nombre de germes | teneur en humidité % |
| 0 | 3000 | 5.1 | 2400 | 5.1 | 15000 | 4.5 | 15000 | 4.5 | 12000 | 5.3 | 12000 | 5.3 |
| 4 | 15000 | 9.1 | 2500 | 8.7 | 3000 | 7.8 | — | 8.8 | — | — | — | — |
| 8 | 16000 | 9.9 | 3200 | 10.0 | 4000 | 8.0 | 7000 | 10.8 | — | — | — | — |
| 13 | 4000 | 9.3 | 4500 | 11.8 | 26000 | 10.0 | 6000 | 12.2 | 3700 | — | 17000 | — |
| 17 | 19000 | 16.1 | 1000 | — | 15000 | 10.8 | 3000 | 11.9 | — | — | — | — |
| 21 | 30000 | 18.8 | 60000 | 16.8 | 6000 | 11.8 | — | 11.8 | — | — | — | — |
| 25 | moisie | 19.8 | moisie | 20.3 | 4500 | 12.3 | — | 11.8 | — | — | — | — |
| 29 | — | — | — | — | 4500 | 11.5 | 3500 | 11.0 | 1200 | — | 3500 | — |
| 34 | — | — | — | — | — | 12.4 | — | 11.2 | — | — | — | — |
| 38 | — | — | — | — | 5000 | 13.9 | 7000 | 13.1 | — | — | — | — |
| 42 | — | — | — | — | 2000 | 13.7 | 5000 | 12.9 | 500 | — | 1000 | — |
| 46 | — | — | — | — | 8000 | 14.4 | 1500 | — | — | — | — | — |
| 53 | — | — | — | — | moisie | 14.8 | — | — | 300 | — | 1200 | — |
| 71 | — | — | — | — | — | — | 1500 | 12.8 | 200 | — | 600 | — |
| 78 | — | — | — | — | — | — | 1500 | 13.6 | — | — | — | — |
| 82 | — | — | — | — | — | — | 3000 | 14.6 | — | — | — | — |
| 87 | — | — | — | — | — | — | 14000 | 13.3 | 1700 | — | 2600 | — |
| 92 | — | — | — | — | — | — | 1500 | 14.5 | — | — | — | — |
| 95 | — | — | — | — | — | — | 10000 | 13.5 | — | — | — | — |
| 100 | — | — | — | — | — | — | moisie | 13.5 | 500 | — | 1500 | — |
| 114 | — | — | — | — | — | — | — | — | 200 | 10.4 | 5000 | 6.1 |
| 128 | — | — | — | — | — | — | — | — | 300 | — | 200 | — |
| 142 | — | — | — | — | — | — | — | — | 1000 | — | 2600 | — |
| 170 | — | — | — | — | — | — | — | — | 3900 | — | 1100 | — |
| 184 | — | — | — | — | — | — | — | — | 1700 | 12.8 | 2400 | 6.7 |
| 212 | — | — | — | — | — | — | — | — | 1000 | 11.5 | 1000 | 6.1 |
| 231 | — | — | — | — | — | — | — | — | 600 | 11.1 | 2000 | 6.3 |
| 255 | — | — | — | — | — | — | — | — | — | 11.1 | — | 6.1 |

évident qu'ici „couche supérieure" a un sens très élastique et qu'une fois la couche était plus épaisse qu'une autre fois.

Les fluctuations du nombre de germes s'expliquent partiellement par la cause mentionnée ci-dessus. Toutefois l'inexactitude de la détermination même joue ici un grand rôle. En mélangeant de l'eau stérile avec de la poudre peu soluble on obtient difficilement un mélange homogène. Aussi c'est la plus grande difficulté que nous ayons eue en ce qui concerne la poudre Hatmaker. Malgré les inexactitudes mentionnées ci-dessus nous pouvons cependant tirer quelques conclusions de nos recherches.

Comme on pouvait s'y attendre, la poudre moisit d'autant plus vite que le degré d'humidité de l'air dans lequel on la conserve est plus élevé. L'essai fait à une teneur en humidité relative de 85% indiquée dans le tableau I fait une exception inexplicable à ce sujet. Les laits secs qui ont fait l'objet de nos recherches ne moisissaient pas même après un temps assez long de conservation à une humidité relative de l'air ambiant de 75% et de 30% quoique la teneur en humidité à 75% se fût assez élevée.

La teneur en humidité dans laquelle la moisissure se produit semble être dépendante du degré d'humidité de l'air dans lequel la poudre est conservée. Dans le tableau III, nous avons réuni les teneurs en humidité où commence la moisissure.

Tableau III.

| Sorte de lait sec | teneur en humidité relative de l'air | | | |
|---|---|---|---|---|
| | 100% | 90% | 85% | 80% |
| Krause | 18.4% | 15.9% | 13.8% | 12.9% |
| Hatmaker | 19.8% | 20.3% | 14.8% | 13.5% |

On y constate que cette teneur en humidité est d'autant plus haute, que la teneur en humidité relative de l'air dans lequel on conserve la poudre est plus élevée.

Le tableau III montre clairement, du moins en ce qui concerne les échantillons que nous avons examinés, que la poudre Hatmaker ne moisit qu'à un degré d'humidité plus élevé que la poudre Krause. En comparant les tableaux I et II il semble bien probable, que la poudre Hatmaker atteint ce degré d'humidité plus vite que la poudre Krause.

En conservant le lait sec, le nombre de germes baisse jusqu'à un certain niveau, assez bas, où il se maintient plus ou moins jusqu'à ce qu'une hausse se produise, à une certaine teneur en humidité. Nous pourrions appeler ce point d'humidité la „teneur en humidité critique".

Nous avons exprimé les données du tableau I d'après Richter-Altschäffer (1) par des courbes correspondant aussi bien que possible aux constatations (voir fig. 1, 2, 3 et 4).

En ce qui concerne les résultats on remarquera ce qui suit. Les courbes indiquant le nombre de germes suivent toutes le même cours. Le premier tournant, terminant la première baisse

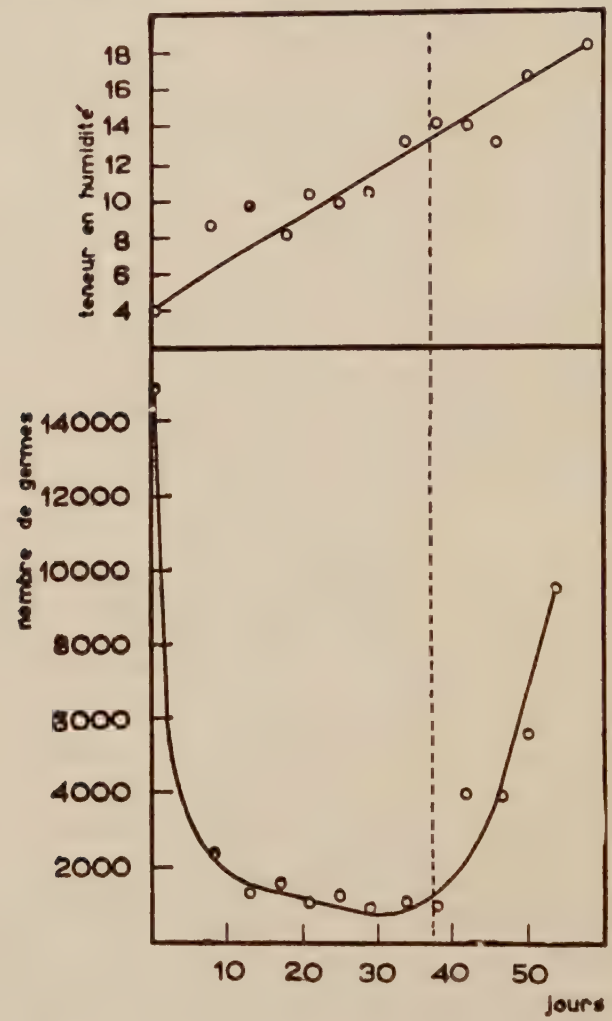


Fig. 1. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 100% d'humidité relative.

rapide du nombre de germes, est atteint: à 100% après 10 jours environ, à 90% après 10 jours environ, à 85% après 17 jours environ et à 80% après 17 jours environ.

Ensuite le nombre de germes reste assez constant jusqu'au moment où une hausse accentuée se produit tout à coup, à 100%

après 37 jours environ, à 90% après 38 jours environ, à 85% après 93 jours environ et à 80% après 68 jours environ.

Si nous considérons le degré d'humidité que la poudre a atteint au moment où la hausse du nombre de germes se produit, on

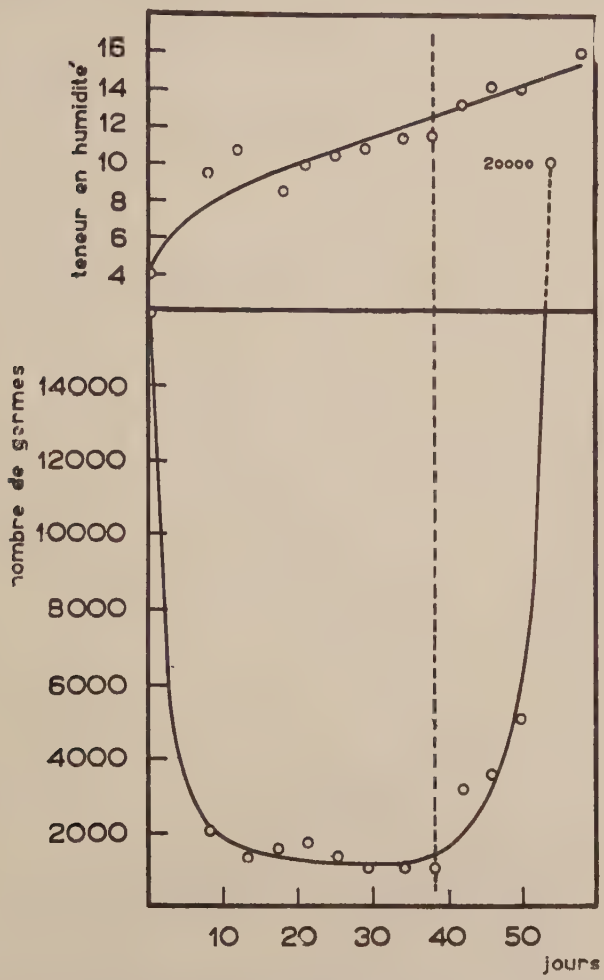


Fig. 2. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 90% d'humidité relative.

constate que ce degré d'humidité à 100% s'est élevé à 13.5%, à 90% s'est élevé à 12.5%, à 85% s'est élevé à 13.5% et à 80% s'est élevé à 12.5%.

Ces courbes nous montreraient donc que la hausse soudaine, indiquée ci-devant, du nombre de germes dans la poudre Krause,

se produit à une teneur en humidité déterminée, indépendamment des circonstances extérieures. Cela n'est pas nécessairement en contradiction avec ce que nous avons fait remarquer plus haut au sujet du degré d'humidité de la poudre en moisissure, puisque

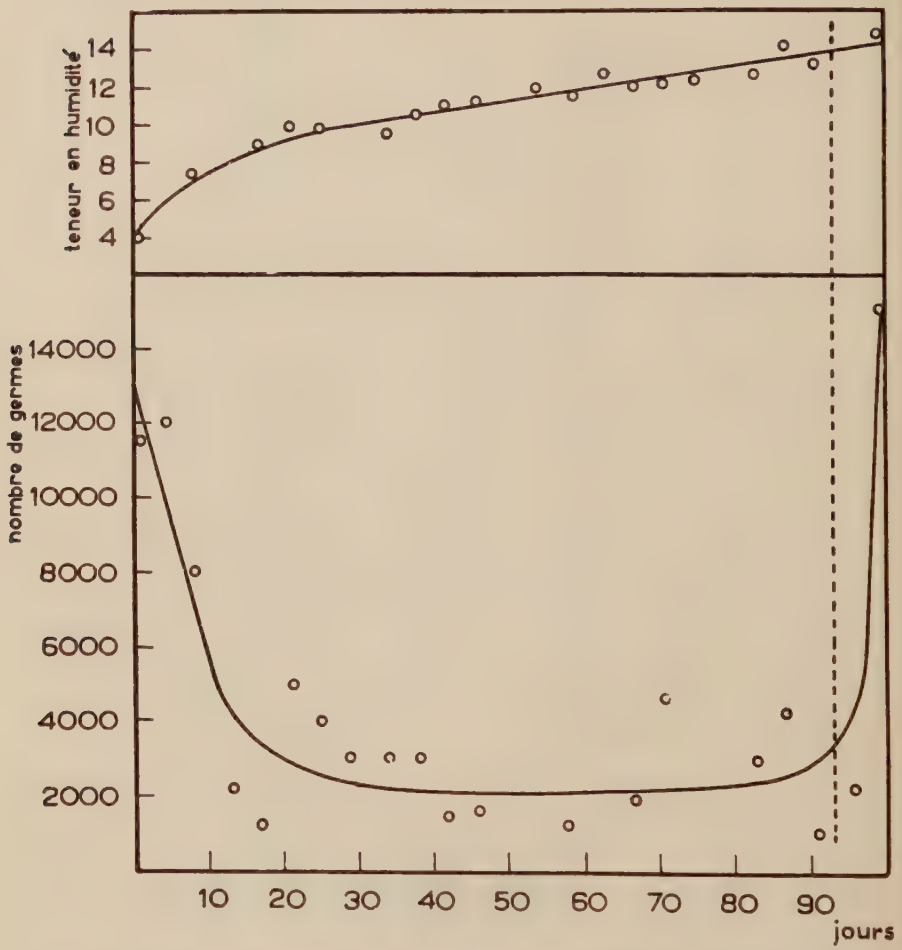


Fig. 3. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 85% d'humidité relative.

pour la production d'une moisissure visible il faut qu'une certaine quantité minimum de mycélium (avec conidies) soit formée. La rapidité du développement du mycélium dépendra certainement de la teneur en humidité relative de l'air ambiant, comme SCHWARTZ et KAESZ (2) l'ont constaté dans des expérimentations concernant le développement des moisissures sur la viande.

3. Reprise de l'expérimentation avec des poudres Krause de fabrication différente.

Des expérimentations susmentionnées nous avons conclu que, très probablement, il existe pour le lait sec une „teneur en humidité critique", c'est à dire une teneur dans laquelle une flore microbienne

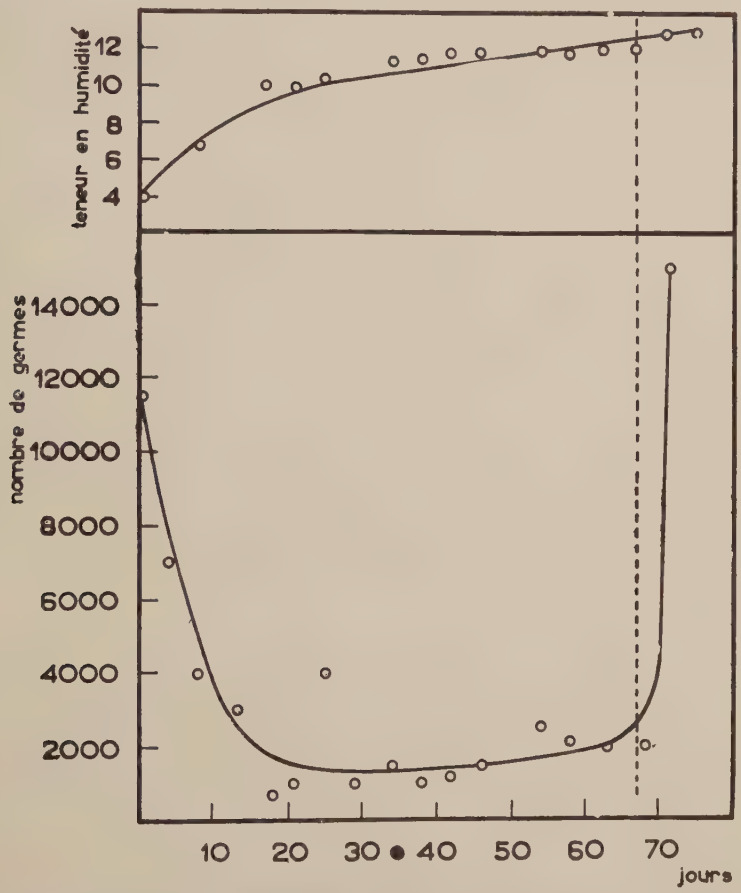


Fig. 4. Cours de la teneur en humidité et du nombre de germes pour la poudre Krause maigre conservée à l'air contenant 80% d'humidité relative.

se développe très rapidement ce qui fait moisir la poudre en peu de temps. Au-dessous de cette teneur le nombre de germes reste constant ou diminue. Nous avons trouvé des indications qui portent à croire que la „teneur en humidité critique" d'une poudre est indépendante de la tension de vapeur relative sous laquelle cette poudre est conservée. Le degré d'humidité de la poudre où celle-ci a commencé à moisir visiblement s'est bien trouvé dépendant

de la tension de vapeur relative du milieu où on conservait la poudre.

Les recherches susdites n'ont été effectuées qu'avec une seule sorte de poudre Krause et de Hatmaker. Comme il n'était pas inimaginable que des poudres de fabriques différentes et produites à diverses époques donneraient des résultats différents, nous avons complété nos recherches par une série d'expériences avec cinq ou six poudres Krause provenant de diverses fabriques. De plus, il était intéressant d'établir l'équilibre de la teneur en humidité sous une certaine tension de vapeur relative. En conservant la poudre en une couche épaisse il s'écoulera beaucoup de temps avant qu'un équilibre ne se soit établi. C'est pourquoi nous avons procédé dans ces expériences en conservant la poudre en couches minces. Cela a, de plus, l'avantage que la reprise d'eau est plus rapide de sorte que la durée de l'expérience est plus courte.

Nous avons examiné les phénomènes qui se produisaient sous une tension de vapeur relative de 100% (au-dessus de l'eau), de 90% (au-dessus d'une solution saturée de $Na_2SO_4$), de 85% (au-dessus d'une solution saturée de KCl) et de 75% (au-dessus d'une solution saturée de NaCl). Dans les trois premières expériences susdites on a procédé comme suit. On a pesé environ 6 g de poudre dans une boîte de Petri. Cinq boîtes de Petri ont été placées l'une au-dessus de l'autre dans un exsiccateur où on avait mis respectivement de l'eau et la solution saturée de sel. Les exsiccateurs étaient placés dans l'obscurité et à la température du laboratoire. A des moments donnés tout le contenu d'une boîte a fait l'objet de recherches sur le degré d'humidité et le nombre de germes.

Les expérimentations sur la conservation à 75% d'humidité relative avaient un caractère un peu autre. Vu les résultats obtenus jusqu'ici, on ne s'attendait pas à ce que les poudres moisissent. C'est pourquoi nous avons seulement suivi le cours de la teneur en humidité tout en examinant surtout s'il s'établirait un état d'équilibre et comment se comporterait la teneur en humidité des diverses poudres dans cet état d'équilibre. La poudre était conservée dans l'obscurité et dans des cuvettes de porcelaine de 7 cm de diamètre. On en pesait exactement 2 grammes de sorte qu'ici, il s'agissait aussi d'une couche mince. La température de conservation était de 22° C. Les cuvettes étaient d'abord pesées tous les quatre jours, plus tard, à de plus grands intervalles. On calculait la teneur en humidité de la hausse d'équilibre trouvée. L'expérience a été faite en double.

Lorsque, au bout de deux mois, on constatait, ici encore, la présence de moisissure on a déterminé également le nombre de germes de ces poudres, mais seulement au moment où le commencement de la moisissure était visible à l'oeil nu. En même temps, comme contrôle, on déterminait l'humidité de la manière ordinaire.

Résultats des recherches.

I. Expériences à 100% d'humidité relative.

Dans les expériences précédentes nous avons trouvé qu'à une humidité relative de 100% la poudre moisissait au bout de deux mois. C'est pourquoi il nous a semblé d'abord suffisant de déterminer la teneur en humidité et le nombre de germes une fois par semaine. Au bout d'une semaine nous avons déja pu constater dans une poudre un accroissement de moisissure tandis qu'au bout de 14 jours la plupart des poudres étaient complètement moisies. Le tableau suivant nous donne le résultat des déterminations de la teneur en humidité et du nombre de germes.

Tableau IV.

| 100% d'humidité relative | A | | B | | C | | D | | Poudre Krause de l'expérience précédente | |
|---|---|---|---|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| tat du début | 2.7 | 53000 | 2.4 | 5900 | 4.0 | 9300 | 3.1 | 478000 | 5.9 | 12200 |
| près 7 jours | 23.6 | 466000 | 21.8 | 3600 | 20.3 | 2200 | 15.3 | 68000 | 13.7 | 1500 |
| près 14 jours | 26.8 | 21000 [1] | 23.0 | 6400 | 21.1 | 56000 | 21.6 | 135000 | 14.9 | 2900 |
| près 21 jours | | [2] | 26.9 | 35000 [1] | 24.9 | [2] | 24.9 | [2] | 17.3 | 181000 |
| près 25 jours | — | — | — | — | — | — | — | — | 20.0 | [1] |

[1]) commencement de moisissure. [2]) moisie complètement.

Tableau IV (suite).

| 100% d'humidité relative | E | | F | | G | | H | |
|---|---|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| Etat du début | 2.6 | 29000 | 6.0 | 24000 | 2.9 | 31500 | 4.1 | 33700 |
| Après 7 jours | 21.4 | 18800 | 23.5 | 170000 | 23.5 | 56000 [1] | 21.5 | 271000 |
| Après 14 jours | 24.9 | 66000 [1] | 25.2 | 124000 [1] | 24.9 | [2] | 28.5 | [2] |

[1]) commencement de moisissure. [2]) couverte de moisissure.

Par ces données on ne peut pas voir le cours du nombre de germes dans la conservation à 100% d'humidité relative. Il y a un indice que le nombre de germes diminue d'abord, ici également. Dans certains cas cependant on pouvait constater une augmentation au bout d'une semaine déjà, ce qui indiquait un commencement de moisissure.

Le moment où la moisissure se produit n'a pas été constaté exactement. Selon ces déterminations nous ne trouvons pas de

grandes différences dans les valeurs concernant la teneur en humidité de la poudre qui vient de moisir. Les valeurs vont de 26.8 à 23.5 pour les nouveaux échantillons examinés; la poudre Krause qui a été employée dans l'expérience précédente faisait seule exception. Le temps qui s'écoule avant que la moisissure se produise était, ici encore, plus long, ce qui permettait de constater plus exactement le moment où la moisissure se produisait. Il est possible que l'absorption d'humidité plus lente vienne de ce que la poudre était plus vieille.

L'expérience a été reprise. Cette fois-ci on a effectué les déterminations tous les quatre jours. On a restreint le nombre de poudres examinées. Le tableau V nous en donne le résultat.

Tableau V.

| 100% d'humidité relative | A | | B | | C | |
|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| Etat du début | 3.2 | 17500 | 3.2 | 5700 | 4.0 | 3700 |
| Après 3 jours | 16.5 | 8000 | 18.9 | 1900 | 14.2 | 1900 |
| Après 6 jours | 15.5 | 7000 | 19.4 | 2600 | 14.0 | 1300 |
| Après 9 jours | 19.9 | 8000 | 21.3 | 2200 | 20.1 | 700 |
| Après 13 jours | 20.9. | 7200 | 20.8 | 3200 | 17.8 | 500 |
| Après 18 jours | — | — | 30.8 | [2]) | — | — |
| Après 19 jours | — | — | — | — | 24.8 | [1]) |
| Après 21 jours | 28.8 | [2]) | — | — | — | — |

[1]) commencement de moisissure. [2]) moisie complètement.

Tableau V (suite).

| 100% d'humidité relative | D | | G | | F | |
|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| Etat du début | 3.6 | 290000 | 3.8 | 22500 | 6.1 | 15400 |
| Après 3 jours | 19.6 | 63000 | 14.8 | 8100 | 21.6 | 9000 |
| Après 6 jours | 14.7 | 66300 | 13.8 | 1800 | 16.3 | 1500 |
| Après 9 jours | 21.8 | 58200 | 20.3 | 16500 | 20.9 | 4700 |
| Après 13 jours | 22.7 | 22100 | 18.2 | 10800 | 23.2 | 1500 |
| Après 18 jours | 33.5 | [1]) | — | — | — | — |
| Après 19 jours | — | — | 18.8 | [1]) | — | — |
| Après 21 jours | — | — | — | — | 33.4 | [1]) |

[1]) moisie complètement.

Par les données de ce tableau on ne peut pas déterminer exactement la teneur en humidité où la poudre moisit. Le degré d'humidité des différentes poudres où l'on a constaté la moisissure dif-

férait et allait de 18.8 à 33.5%. La valeur de 18.4% trouvée dans l'expérience précédente est donc probablement dans les circonstances données une valeur minimum.

II. Expériences à 90% d'humidité relative.

Ces expériences ont été faites tout à fait de la même manière. Seulement, ici comme dans l'expérience à 85% d'humidité, il a été possible d'examiner la poudre une fois par semaine. L'absorption d'humidité avait lieu ici beaucoup plus graduellement que dans une atmosphère plus humide.

Tableau VI.

| 90% d'humidité relative | A | | B | | C | | D | |
|---|---|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| Etat du début | 3.2 | 46400 | 4.7 | 4800 | 7.9 | 1800 | 4.0 | 270000 |
| Après 8 jours | 20.2 | 15200 | 23.2 | 23400 | 20.2 | 5800 | 22.4 | 18400 |
| Après 14 jours | 21.4 | 7100 | 24.8 | 3300 [2]) | 21.0 | 2000 [2]) | 20.4 | 20000 [1]) |
| Après 21 jours | 26.8 | 8500 [1]) | — | — | — | — | — | — |

[1]) commencement de moisissure. [2]) moisie.

Les résultats du tableau VI nous montrent qu'il est probable que la teneur en humidité où la moisissure se produit à 90% d'humidité relative est plus basse qu'à 100%. Les valeurs obtenues pour cette teneur sont passablement plus élevées que les valeurs trouvées dans l'expérience précédente (voir tableau I).

III. Expériences à 85% d'humidité relative.

Les résultats de ces expériences sont réunis dans le tableau VII.

Tableau VII.

| 85% d'humidité relative | A | | B | | C | | D | |
|---|---|---|---|---|---|---|---|---|
| | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes | humidité % | nombre de germes |
| Etat du début | 3.2 | 46400 | 4.7 | 4800 | 7.9 | 1800 | 4.0 | 270000 |
| Après 8 jours | 14.6 | 8200 | 14.6 | 3400 | 11.6 | 1200 | 14.4 | 54000 |
| Après 15 jours | 15.5 | 7900 | 15.7 | 3800 | 13.3 | 1200 | 13.3 | 7000 |
| Après 22 jours | 16.3 | 6400 | 15.4 | 3300 | 13.2 | 600 | 15.4 | 19600 |
| Après 27 jours | 16.6 | 3700 [1]) | 16.0 | 5200 [1]) | — | — | 15.9 | 9800 [1]) |
| Après 36 jours | — | — | — | — | 14.3 | 1800 | — | — |
| Après 38 jours | — | — | — | — | 14.4 | 3700 [1]) | — | — |

[1]) commencement de moisissure.

De ces données nous pouvons conclure, que la teneur en humidité où se produit la moisissure est environ de 16%. Selon la méthode décrite précédemment, avec une couche épaisse de poudre, nous avons trouvé une valeur plus basse (voir tableau III).

Au sujet de la marche du nombre de germes nous pouvons remarquer qu'ici, de nouveau, une baisse a d'abord été constatée.

IV. Expériences à 75% d'humidité relative.

Comme nous l'avons exposé ci-dessus, ces expériences ont été

Tableau VIII.

| 75% d'humidité relative | A | | B | | C | | D | | E | |
|---|---|---|---|---|---|---|---|---|---|---|
| Etat du début | 3.0 | 3.0 | 2.4 | 2.4 | 3.5 | 3.5 | 3.1 | 3.1 | 2.6 | 2.6 |
| Après 4 jours | 13.2 | 12.8 | 21.6 | 20.2 | 12.5 | 12.4 | 11.8 | 11.9 | 18.6 | 19.1 |
| Après 8 jours | 12.9 | 12.7 | 18.4 | 16.8 | 12.5 | 12.4 | 11.8 | 12.2 | 16.6 | 17.1 |
| Après 11 jours | 12.9 | 12.4 | 16.5 | 15.3 | 12.4 | 12.4 | 11.7 | 12.5 | 14.9 | 15.0 |
| Après 17 jours | 13.3 | 12.7 | 13.6 | 12.7 | 12.2 | 12.9 | 12.2 | 12.6 | 13.1 | 13.3 |
| Après 20 jours | 13.1 | 12.8 | 13.5 | 12.8 | 12.4 | 12.6 | 11.8 | 12.5 | 12.5 | 12.4 |
| Après 24 jours | 13.2 | 13.9 | 13.6 | 12.9 | 12.6 | 12.6 | 11.9 | 12.6 | 13.0 | 12.9 |
| Après 28 jours | 13.1 | 12.7 | 13.7 | 12.9 | 12.6 | 12.4 | 12.0 | 12.7 | 12.7 | 12.3 |
| Après 34 jours | 13.2 | 13.0 | 13.3 | 12.9 | 12.7 | 12.6 | 11.7 | 13.1 | 12.7 | 12.5 |
| Après 50 jours | 12.6 | 12.7 | 13.2 | 12.3 | 12.1 | 12.4 | 11.7 | 12.2 | 12.0 | 12.3 |
| Après 56 jours | 12.6 | 11.8 | — | — | 11.1 | 10.9 | — | — | — | — |
| Après 57 jours | 12.6 | 12.3 | — | — | 12.0 | 11.7 | — | — | — | — |
| Après 58 jours | — | — | — | — | — | 11.7 | — | — | — | — |
| Après 62 jours | 12.9 | 12.0 | — | — | 12.2 | — | — | — | — | — |
| Après 65 jours | 13.0 | — | — | — | — | — | — | — | — | — |
| Après 68 jours | — | — | — | — | — | — | — | — | — | — |

| 75% d'humidité relative | F | | G | | H | | Poudre Krause expérience précédente | |
|---|---|---|---|---|---|---|---|---|
| Etat du début | 6.0 | 6.0 | 2.9 | 2.9 | 4.1 | 4.1 | 5.9 | 5.9 |
| Après 4 jours | 14.8 | 14.9 | 12.1 | 12.0 | 20.5 | 21.1 | 12.8 | 13.6 |
| Après 8 jours | 15.9 | 15.1 | 14.2 | 12.0 | 18.0 | 18.2 | 12.5 | 14.1 |
| Après 11 jours | 16.4 | 14.8 | 13.5 | 12.2 | 15.6 | 15.7 | 12.8 | 14.3 |
| Après 17 jours | 16.6 | 14.9 | 13.6 | 12.3 | 12.4 | 12.8 | 12.8 | 13.9 |
| Après 20 jours | 16.4 | 14.6 | 13.4 | 11.8 | — | — | 12.8 | 13.9 |
| Après 24 jours | 16.4 | 14.4 | 13.6 | 12.2 | — | — | 13.1 | 13.8 |
| Après 28 jours | 14.6 | 13.7 | 13.7 | 12.1 | — | — | 13.0 | 13.2 |
| Après 34 jours | 15.3 | 14.8 | 14.3 | 12.6 | — | — | 12.9 | 13.2 |
| Après 50 jours | 15.3 | 14.0 | 13.6 | 12.2 | — | — | 12.8 | 13.5 |
| Après 56 jours | 12.0 | 12.4 | 13.6 | 11.8 | — | — | 13.0 | 13.3 |
| Après 57 jours | 14.2 | 13.9 | 13.3 | 12.4 | — | — | 12.4 | 12.9 |
| Après 58 jours | — | — | — | — | — | — | 12.2 | 12.8 |
| Après 62 jours | 14.2 | — | 13.5 | 12.2 | — | — | — | — |
| Après 65 jours | — | — | 13.7 | — | — | — | — | — |
| Après 68 jours | — | — | 13.2 | — | — | — | — | — |

faites pour déterminer la teneur en humidité dans l'état d'équilibre. Cette teneur a été calculée au moyen de la hausse de poids mesurée. Les résultats obtenus sont donnés en double dans le tableau VIII. Dans la fig. 5 nous donnons comme exemples caractéristiques, la marche de la teneur en humidité dans les poudres A et B.

Les valeurs indiquées dans le tableau sont celles qu'on a constatées jusqu'au début de la moisissure; dans le graphique on trouve la marche de la teneur en humidité pendant 50 jours.

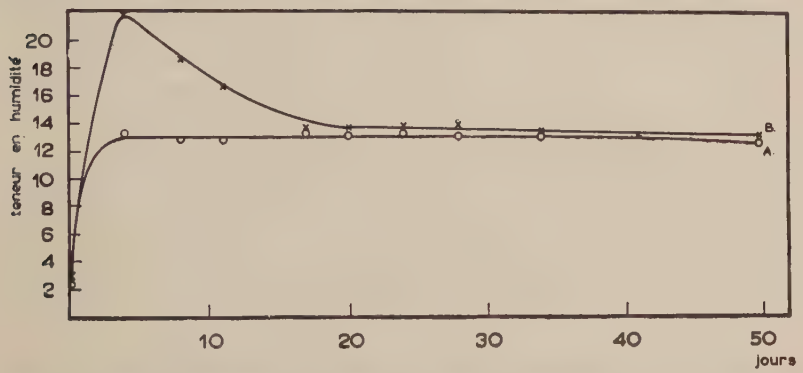


Fig. 5. Cours de la teneur en humidité pour les poudres A et B conservées à l'air contenant 75% d'humidité relative.

Dans le tableau IX nous donnons l'état final des poudres. A côté de la teneur en humidité, calculée d'après la hausse de poids trouvée, nous donnons les valeurs pour la teneur que nous avons trouvée par une détermination directe en séchant la poudre conservée jusqu'au poids constant à 103° C.

Si nous portons d'abord notre attention sur les résultats obtenus en ce qui concerne la teneur en humidité des poudres en état d'équilibre, alors nous remarquons en premier lieu qu'en effet un tel équilibre est atteint. Les valeurs obtenues pour cette teneur en humidité dans les différentes poudres varient de 14.2 à 11.7%. Nous n'avons donc pas trouvé de grandes différences.

Nous avons bien trouvé une différence dans la manière de laquelle cet état d'équilibre de la teneur en humidité a été atteint. Les poudres B, E et H avaient un caractère à part. Tandis que les autres poudres avaient déjà atteint leur maximum d'humidité au bout de 4 jours et qu'en les conservant plus longtemps elles restaient assez constantes, ces poudres (B, E et H) montraient une assez grande hausse pendant les 4 premiers jours et rebaissaient fort ensuite en atteignant une valeur conforme à celle des autres poudres. Supplee et Tamsma ont également constaté ce sommet dans la courbe, dans la conservation à 50%, ou plus, d'humidité relative.

Naturellement, il est possible que les autres poudres aient marqué un tel sommet, mais en tout cas elles étaient déjà redes-

Tableau IX.

| 75% d'humidité relative | durée de conservation (en jours) | teneur d'humidité | | nombre de germes | |
|---|---|---|---|---|---|
| | | calculée | déterminée | bactéries | moisissures |
| A | 65 | 13.0 | 10.1 | 9000 | 300 |
| | 62 | 12.0 | — | 5600 | 1300 |
| B | 50 | 13.2 | 11.7 | 1700 | 3200 |
| | 50 | 12.3 | 12.1 | 1400 | 6900 |
| C | 65 | 12.2 | 9.9 | 600 | 8400 |
| | 58 | 11.7 | 11.0 | 1100 | 7100 |
| D | 50 | 11.7 | 10.5 | 1900 | 0 |
| | 50 | 12.2 | 10.2 | 1200 | innombrables |
| E | 50 | 12.0 | 9.8 | 1700 | 200 |
| | 50 | 12.3 | — | 1600 | 0 |
| F | 62 | 14.2 | 10.9 | 2500 | 3800 |
| | 57 | 13.9 | 10.2 | 2300 | 3800 |
| G | 68 | 13.2 | 11.3 | 1100 | 200 |
| | 62 | 12.2 | 12.2 | 300 | 400 |
| H | 17 | 12.4 | — | — | — |
| | 17 | 12.8 | 14.5 | — | — |
| Poudre Krause de l'expérience précédente | 58 | 12.2 | 10.8 | 1200 | 5000 |
| | 58 | 12.8 | 11.2 | 1700 | 300 |

cendues au point d'équilibre de la teneur en humidité au bout de 4 jours. Cette possibilité est même très probable, vu les constatations de SUPPLEE, qui a trouvé le maximum à 50% après 33 heures.

Enfin, quelques mots sur le début de la moisissure à 75% d'humidité relative.

La poudre H marquait à cet égard le plus grand écart. Elle était déjà moisie complètement après 17 jours tandis que pour les autres poudres, le temps qui s'écoulait avant que la moisissure fût constatable, variait de 50 à 68 jours.

Par cette expérience nous ne voyons pas d'une manière évidente pourquoi, dans ces cas, la moisissure ne se produit que si tard. Comme nous l'avons déjà dit, dans la plupart des cas la teneur en humidité définitive est déjà atteinte au bout de 4 jours. Le dit phénomène sera probablement en rapport avec le fait que la plupart des moisissures à 75% d'humidité relative ne se développent pas, ou seulement très lentement. Cette poudre, moisissant plus vite, aurait alors contenu des spores d'une sorte particulièrement xerophile. La teneur en humidité où se produit la moisissure à

75% d'humidité relative, se trouve encore beaucoup plus basse que la teneur en humidité, correspondant à une humidité relative plus élevée et se rapproche plus de la „teneur en humidité critique" dont nous avons parlé au commencement du paragraphe 4.

Il faut attirer l'attention sur le fait qu'il y a une assez grande différence entre la teneur en humidité calculée d'après la hausse de poids et la teneur déterminée de la manière ordinaire. En laissant la poudre H à part [1]), alors, nous voyons que la teneur en humidité déterminée de la manière directe est toujours plus basse que celle qui a été calculée. Cela pourrait indiquer qu'une partie de l'eau absorbée est si intimement liée qu'elle ne se dégage plus lorsqu'elle est chauffée à 103° C.

La détermination du nombre de germes montre qu'à la suite d'une si longue conservation le nombre de bactéries est à peu près égal dans toutes les poudres. Ce nombre de bactéries est très réduit. Le nombre de moisissures trouvées est assez arbitraire. Une différence importante entre les expériences précédentes faites dans une humidité relative plus élevée et cette dernière c'est que dans celle-ci l'équilibre de la teneur en humidité s'atteint en premier lieu et que ce n'est qu'après que la moisissure se produit. Par ces circonstances il est plus facile de déterminer au juste la teneur en humidité au commencement de la moisissure. Dans les teneurs en humidité relative plus élevées il semble qu'au début de la moisissure l'équilibre dans l'état d'humidité de la poudre n'est pas encore atteint. Ici la poudre moisit déjà lorsqu'on peut s'attendre à une hausse importante dans la teneur en humidité. Le développement de la moisissure empêche ici la détermination de la teneur en humidité dans l'état d'équilibre.

4. Relation entre la teneur en humidité où se produit la moisissure et la tension de vapeur relative de l'ambiance.

Dans les expériences de conservation en couches minces nous avons constaté que la teneur en humidité où se produit la moisissure dépend aussi de l'humidité relative du milieu où la poudre est conservée. Suivant le tableau X, où sont réunies ces teneurs en humidité, ce degré d'humidité est d'autant plus haut que l'humidité relative est plus élevée.

Il est admissible que cette dépendance difficile à comprendre, à première vue, n'est qu'apparente. En réalité la moisissure pourrait dans tous les cas commencer à une même „teneur en humidité critique". Comme il s'écoule pourtant assez de temps avant que la moisissure en soit au critérium „moisissure visible", il faudra tenir compte que pendant cette période le procès d'absorption d'humidité continue et devient ensuite d'autant plus rapide, par la force des choses, que l'humidité relative du milieu est plus

[1]) Cette poudre était toute moisie lorsque la teneur en humidité était déterminée. Les autres poudres commençaient à moisir lorsqu'on déterminait leur teneur finale.

grande. Les différences dans le développement de la moisissure dans les différentes humidités relatives ne seront pas assez grandes pour compenser les différences dans la vitesse de l'absorption de l'humidité.

Tableau X.

| humidité relative | 100% | 90% | 85% | 75% |
|---|---|---|---|---|
| A | 26 | 25 | 17 | 12 |
| B | 27 | 20 | 16 | 12 |
| C | 25 | 20 | 14 | 13 |
| D | 25 | 22 | 16 | 12 |
| E | 25 | — | — | 12 |
| F | 25 | — | — | 14 |
| G | 24 | — | — | 13 |
| H | 25 | — | — | 12 |
| Poudre Krause de l'expérience précédente | 20 | — | — | 12 |

## Résumé.

1. Les expériences ont été faites dans le but de suivre le cours du nombre de germes et de la teneur en humidité du lait sec en le conservant à différents degrés d'humidité relative. On a commencé par conserver la poudre en couche d'une épaisseur quelconque. Plus tard on a conservé des échantillons de fabrication différente en couches minces.
2. Si on met le lait sec dans un milieu dont il peut absorber l'humidité alors une forte baisse du nombre total de germes a d'abord lieu. Puis ce nombre de germes reste assez constant pendant longtemps pour remonter de nouveau quand l'humidité dans la poudre a atteint un certain niveau. Au-dessous de cette teneur en humidité critique le développement d'une flore microbienne considérable n'est pas possible.
3. La teneur en humidité critique est indépendante de la tension de vapeur de l'air où la poudre a absorbé de l'eau.
4. La teneur en humidité où la moisissure visible se produit est d'autant plus basse que la poudre est conservée dans une humidité relative plus basse.
5. Dans une humidité relative de 100%, 90% et de 85% la moisissure se produit avant que la teneur en humidité de la poudre soit en équilibre avec l'humidité de l'air ambiant; à 75% d'humidité relative l'équilibre s'est déjà établi avant le début de la moisissure.
6. Avant d'atteindre l'équilibre à 75% d'humidité relative quelques poudres ne reprenaient de l'humidité que beaucoup plus tard, jusqu'à une teneur de 20% environ, puis cette teneur rebaissait

et atteignait une valeur normale conforme à celle des autres poudres qui absorbaient l'eau immédiatement jusqu'à cette teneur normale.

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K. C. WINKLER, Iets over de stikstof-stofwisseling bij *Bact. coli* (Aspects of the nitrogen metabolism of *B. coli*). Chem. Weekblad **40**, 147, 1943.

The quotient of the decrease of ammonium in the medium and the number of bacteria present is determined after various intervals in a medium containing ammonium sulphate as sole source of nitrogen on which *B. coli* was cultivated. Along with the increase in age of the cultures the quotient decreases considerably (1000× or more).

As it is impossible that the bacteria would have contained initially a 1000 fold amount of nitrogen, it is to be accepted that at the start a part of the ammonium is changed into amino-nitrogen which is not taken up by the bacteria. This may lead to the supposition that the synthesis of amino acids is an exogenic process located in the membrane of bacteria. Amino acids are probably produced already in the lag phase; perhaps a definite external concentration has to be reached before the synthesis of protein may occur. W. H.

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N. SAVRIJ, Eenige vergelijkende onderzoekingen over het aantoonen van coli-bacteriën in gepasteuriseerde melk (Some comparitive investigations of the means of detecting *B. coli* in pasteurised milk). Chem. Weekblad **38**, 114, 1941.

Compared are: The method of the milk decree 1916 (enrichment in acid broth), the method of an official control instance (enrichment in meat broth with lactose and neutral red and cultures on Endo plates), and the method of the laboratory of the Cooperative Plant of Milk products at Bedum (enrichment in a peptone-lactose-brillant green-gall medium and culture on eosine-methylene blue plates).

The results vary, especially when the fermentation test is negative. It became evident that on the Endo plate red colonies even those with a metallic lustre are often not due to *B. coli*. On the other hand on the eosine-methylene blue plates colonies with a metallic lustre are always formed by bacteria of the coli group.

Therefore it seems desirable to base the research for *B. coli* in pasteurised milk on: 1. the occurrence of gas in a lactose containing medium. 2. the metallic lustre of colonies on eosine-methylene blue plates. W. H.

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A. J. KLUYVER, De microbiologische grondslagen der voedselconserveering (The microbiological basis of the conservation of food). Chem. Weekblad **38**, 383, 1941.

The paper contains chiefly a discussion of the action of high and low temperatures and more especially the harmful action of high temperatures on bacteria. The loss of the reproductional power is

taken as a criterium for the death of the bacteria. By means of graphs the letality of the bacteria and the spores as a function of time at various temperatures is visualised. Moreover of importance are: the original number of microbes, the state of maturity of the spores present, the chemical composition and especially the pH of the surrounding medium. Some results arrived at under practical conditions are discussed.

Besides this the advantages or disadvantages of temperatures beneath and just above the freezing point are discussed. Here as well the initial number of microbes is of importance and also the degree of moistness.

The influence of carbon dioxide, ozonising, drying and conservation by means of salt, sugar and acids is shortly discussed.

W. H.

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L. Bijlmer, Aetiologie der Influenza, De isoleering van het influenza-virus tijdens de epidemie van 1941 te Groningen (Etiology of Influenza. The isolation of the influenza virus during the epidemic of 1941 at Groningen). Diss. Groningen, 1943. H. E. Stenfert Kroese's Uitgevers-Mij N.V., Leiden.

The experimental studies on influenza, during the epidemic of January-February 1941 at Groningen (Holland), were carried out according to the technique evolved by Wilson Smith, Andrewes and Laidlaw, of the National Institute for Medical Research at Hampstead (London). The investigations were done in a laboratory specially built in 1940 for the study of experimental influenza in ferrets and since 1941 incorporated in the Institute for Hygiene and Bacteriology of the State University.

During the period of January 9th to February 7th, material from 13 patients, partly throat-washings, partly suspensions of sputa, obtained during the first days of illness, was inoculated in ferrets, which were kept under rigid measures of isolation. The temperature was read twice daily and the clinical signs were recorded.

In 5 cases the ferrets developed a typical influenza infection, with the characteristic fever peak on the second or third day and catarrhal signs of the nose, sometimes of the conjunctiva. In all these cases influenza antibodies against the virus strain WS could be demonstrated in the ferret convalescent serum by means of the mouse protection test.

In two other cases the signs in the ferrets were doubtful, but the ferret convalescent serum contained antibodies against the influenza virus, so that these animals must be assumed to have gone through a subclinical infection.

In 4 of the 6 remaining cases in which the ferrets did not show any reaction, the patients' convalescent serum neither showed any rise in antibodies. In the remaining 2 both the mouse protection and complement fixation test of the patients' sera gave positive results, so that missed ferret inoculation must be assumed.

Three virus strains were isolated, from three of the enumerated cases. After some ferret passages (3, 4 and 5) the virus was adapted to mice. The strains produced lethal pulmonary lesions in the mice after 9, 3 and 7 passages respectively.

In the course of the ferret and mouse passages the virus suspension was filtered through a collodium membrane (average pore diameter 0.6 $\mu$). The throat-washings and sputa from the patients were not filtered before being inoculated in the ferrets, in order not to reduce the virulence of the pathogenic agent.

The nature of the pulmonary lesions of the mice was checked microscopically by sectioning the lung. Hereby we accepted the criteria described by STRAUB.

The analysis of the three isolated influenza virus strains did not show any mutual antigenic differences. The new strains were not found to be identical with any of the English „specific" strains.

The immunological study of the sera from fifty influenza patients demonstrated, that in 33 cases the influenza virus had been the etiological agent; among these were 13 cases of pneumonia, following an attack of influenza.

Prophylaxy and therapy by means of vaccine and immune serum are discussed. A multivalent anti-influenza serum was prepared by hyperimmunising rabbits with various virus strains. Some prophylactic effect of rabbit immune serum, intranasally administered to mice infected with influenza virus, could be noted.

The aim of this study may not be considered reached by the ascertainment of the etiology in a single influenza epidemic. This work must be seen as a preparation for the study of an influenza pandemic. For the Netherlands the research work in this field is being organised by the Institute for Preventive Medicine (Instituut voor Preventieve Geneeskunde) at Leiden.

L. BIJLMER.